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12. Knopf *et al.* (*5*) have reported that the protein kinases expressed in the COS cells, PKC I and PKC II, which correspond to γ type and βII type, respectively, required an extremely high concentration of Ca^{2+} (3 m*M*) for their maximal enzymatic activities. Both βI and βII enzymes obtained in our studies were activated by physiologically low concentrations of Ca^{2+} as observed for rat brain PKC.
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Expression of the Multidrug-Resistant Gene in Hepatocarcinogenesis and Regenerating Rat Liver

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Preneoplastic and neoplastic liver nodules and hepatocytes isolated from regenerating rat liver have been shown to be resistant to a broad range of carcinogenic agents. This phenomenon was studied by measuring the expression of the multidrug-resistant (*mdr*) gene in normal liver cells and in preneoplastic and neoplastic nodules and regenerating liver. Levels of messenger RNA for the *mdr* gene, which encodes P-glycoprotein, were elevated in both preneoplastic and neoplastic lesions. Expression of the *mdr* gene also reached high levels in regenerating rat liver 24 to 72 hours after partial hepatectomy. These results show that the expression of the *mdr* gene can be regulated in liver and is likely to be responsible for part of the multidrug-resistance phenotype of carcinogen-initiated hepatocytes and regenerating liver cells.

THE HYPOTHESIS THAT EXPOSURE TO chemical carcinogens results in the development of a population of cells resistant to the cytotoxic effects of carcinogens was first proposed almost 50 years ago (*1*). Farber and his colleagues provided data to support the hypothesis that development of the carcinogen-resistant phenotype is an early step in the neoplastic process, possibly coinciding with initiation (*2*). The model system best studied is the induction of rat hepatomas after treatment with chemical carcinogens (*2, 3*). Normal rat hepatocytes in monolayer culture are highly sensitive to the cytotoxic effects of methotrexate, adriamycin, cycloheximide, and aflatoxin B_1, whereas liver cells isolated from carcinogen-treated rats are resistant to the toxic effects of these agents (*3*). Although the mechanisms of this pleiotropic resistance are not known, some data suggest that the intracellular concentration of both the carcinogen and its metabolites is lower in the resistant cell than in the normal hepatocyte (*4*).

One mechanism that is known to lower intracellular drug concentrations is the expression of the *mdr* gene (*5*), which results in multidrug resistance to hydrophobic natural products such as adriamycin, colchicine, the *Vinca* alkaloids, and actinomycin D (*6*). Full-length complementary DNAs for the human and mouse *mdr* genes have been cloned and used to demonstrate that expression of the *mdr* gene, which encodes a 170,000-dalton membrane glycoprotein (P-glycoprotein), is responsible for multidrug resistance (*7*). Sequence analysis indicates that P-glycoprotein has 12 hydrophobic membrane-spanning regions and two nucleotide-binding domains (*8*). Taken together with direct biochemical evidence that P-glycoprotein binds drugs (*9*), these results are consistent with its function as an energy-dependent drug efflux pump.

Because the P-glycoprotein efflux pump can lower intracellular drug concentrations and because the *mdr* gene is known to be expressed at somewhat increased levels in normal human and rodent liver (*10*), we investigated the possibility that a further increase in expression of this gene might be associated with the development of carcinogen resistance during chemical carcinogenesis and in regenerating rat liver. Our results show increased expression of the *mdr* gene in preneoplastic and neoplastic carcinogen-induced nodules compared to expression in normal rat liver and a dramatic increase in *mdr* messenger RNA (mRNA) levels in regenerating rat liver. These data suggest that expression of the *mdr* gene is regulated in liver and represents a component of the hepatic response to some toxic insults.

As has been shown in normal human liver (*10*), *mdr* RNA is readily detectable in normal rat liver (Fig. 1A, lane 7). The expression of the *mdr* gene was increased in both preneoplastic and neoplastic liver lesions produced by the Solt-Farber method (Fig. 1A, lanes 1 to 6). No apparent difference in *mdr* expression was observed between the early preneoplastic lesions (nodules isolated 6 to 8 weeks after initiation, Fig. 1, A and B, lanes 5 and 6) and neoplastic lesions (nodules isolated 6 to 8 months after initiation; Fig. 1A, lanes 1 to 4). Both fetal and adult rat liver showed lower levels of *mdr* RNA (Fig. 1A, lane 7; Fig. 1B, lanes 1 to 4). The Solt-Farber protocol involves only a single initiating dose of a carcinogen (in our case, diethylnitrosamine, 200 mg/kg, intraperitoneally) followed by a short period of exposure to a low dose of another carcinogen [2-acetylaminofluorene (AAF)] during which

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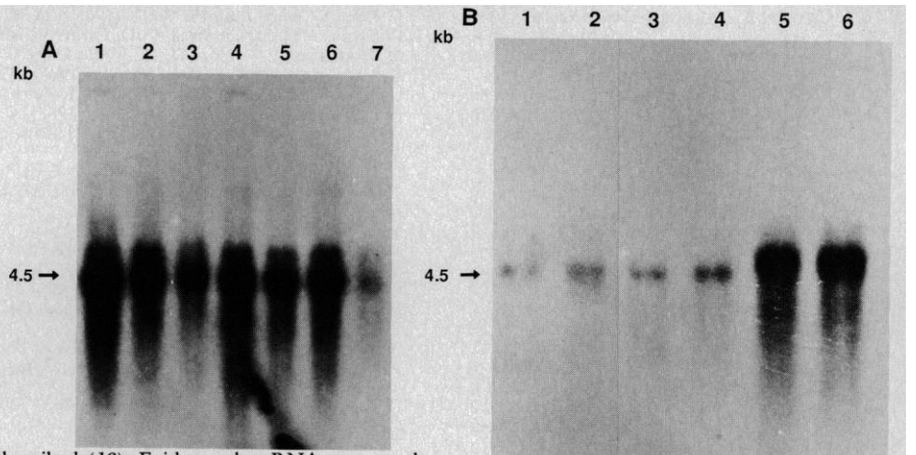

Fig. 1. Transcripts of *mdr* in normal adult and fetal rat liver and during chemical hepatocarcinogenesis. Fetal livers were obtained at 18 days of gestational age from male Fischer rats. Preneoplastic and neoplastic liver lesions were produced by means of the protocol of Solt and Farber (*11*) with minor modification (*15*). Briefly, animals were treated with a single intraperitoneal injection of the initiating agent, diethylnitrosamine (200 mg/kg); after a recovery period of 14 days, animals were administered AAF by gavage (1 mg/day) five times per week for 2 weeks. On day 5 of AAF feeding, animals were subjected to partial hepatectomy according to the method of Higgins and Anderson (*16*) and then continued to receive AAF for an additional week. Hyperplastic nodules (preneoplastic lesions) isolated 6 to 8 weeks after initiation with diethylnitrosamine were pooled (eight to ten nodules per pool) whereas neoplastic nodules (neoplastic lesions representing primary hepatocellular carcinomas) isolated 6 to 8 months after initiation were individually isolated and processed. RNA was isolated with guanidine thiocyanate by the method of Schweizer and Goerttler (*17*) and enriched for polyadenylated [poly(A)] RNA by oligo(dT)-cellulose chromatography (*18*). Electrophoresis of poly(A) RNA samples on horizontal denaturing formaldehyde agarose gels with subsequent transfer to nitrocellulose membranes was performed as described (*19*). Evidence that RNA was not degraded and was equally loaded and transferred to nitrocellulose was obtained by staining the RNA-containing gel with ethidium bromide and observing the gel before and after transfer. The construction of the RNA probe used in all blot hybridization analyses as well as the hybridization conditions were as described (*10*). (**A**) Northern blot analysis of poly(A) RNA isolated from neoplastic liver nodules (lanes 1 to 4), preneoplastic liver nodules (lanes 5 and 6), and normal liver (lane 7). All lanes contained 5 μg of poly(A) RNA. (**B**) Northern blot analysis of poly(A) RNA isolated from fetal rat liver (lanes 1 and 2), adult rat liver (lanes 3 and 4), and preneoplastic liver nodules (lanes 5 and 6). All lanes contain 5 μg of poly(A) RNA.

time a partial hepatectomy is performed. Consequently, the preneoplastic and neoplastic cell populations have only been exposed to the carcinogens for a brief period of time. The contribution of AAF to the development of the initiated cell populations is thought to be the indirect result of growth inhibitory effects of AAF on normal hepatocytes during regeneration after partial hepatectomy (*11*). No preneoplastic cell population develops if the initiating dose of diethylnitrosamine is omitted. The increased expression of multidrug-resistant liver cells is not likely to be due to selection of multidrug-resistant liver cells during the carcinogenesis process, since multidrug-resistant KB carcinoma cells (*12*) expressing high levels of *mdr* mRNA are not cross-resistant to either diethylnitrosamine or AAF (*13*).

The expression of *mdr* mRNA was also examined after partial hepatectomy (Fig. 2). No increase in *mdr* expression was observed for the first 12 hours after partial hepatectomy (Fig. 2, lanes 1 to 3). However, *mdr* mRNA rose to a high level from 24 to 72 hours after hepatectomy, which was at least tenfold as high as *mdr* mRNA levels in control liver (Fig. 2, lanes 4 to 6) and fell 120 hours after hepatectomy (Fig. 2, lane 7). The same results were obtained in a second independent experiment. The level of *mdr* expression seen in these studies is comparable to the high levels seen in human adrenal glands or in multidrug-resistant cell lines that show 100-fold increased resistance to colchicine, vinblastine, and adriamycin (*12*). The increased levels can be due to increased transcription of the gene or decreased degradation of *mdr* mRNA. The decline in *mdr* mRNA levels between 72 and 120 hours after hepatectomy (Fig. 2, lanes 6 and 7) is consistent with a relatively short half-life for the *mdr* mRNA under some conditions.

Our results demonstrate modulation of *mdr* expression under conditions other than selection of multidrug-resistant cell lines after exposure to chemotherapeutic agents and/or chemical carcinogens. Moreover, an experimental model system now exists that offers exciting possibilities for determining the role and mode of regulation of the *mdr* gene in normal cell biology.

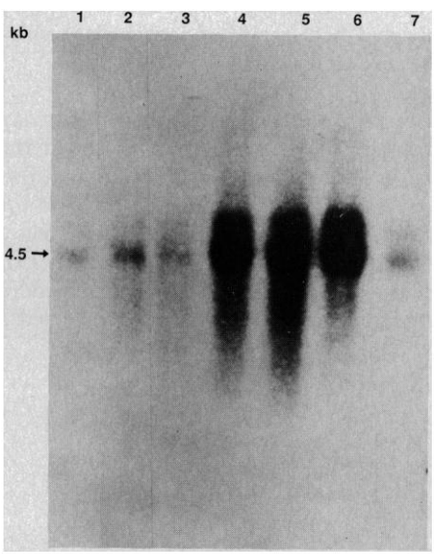

Fig. 2. Transcripts of *mdr* in regenerating rat liver. Poly(A) RNA was isolated at various times after partial hepatectomies of adult male Fischer rats (for details see legend to Fig. 1). Lanes 1 to 7 contain 5 μg of poly(A) RNA isolated at 1, 6, 12, 24, 48, 72, and 120 hours after partial hepatectomy, respectively.

As to the role of the *mdr* gene in the initiation stage of chemical carcinogenesis, a number of questions need to be answered. It is unlikely that the increased expression of the *mdr* gene observed in both preneoplastic and neoplastic cell populations can totally explain the resistance to chemical carcinogens observed in early initiated cells, since many of these carcinogens are compounds not usually affected by the multidrug-resistance phenotype. In addition, if increased expression of the *mdr* gene during the time period of maximal DNA synthesis after partial hepatectomy made liver cells resistant to the cytotoxic and mitoinhibitory effect of AAF, then the Solt-Farber protocol would not result in formation of liver nodules. Therefore, other factors, such as the overexpression of glutathione transferases and other unknown mechanisms, may be contributing significantly to the development of the resistance phenotype (*14*). If this is the case, the regulated expression of the *mdr* gene may be part of a programmed set of responses of the liver cell to certain toxic injuries.

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In Vivo Uncoating and Efficient Expression of Foreign mRNAs Packaged in TMV-Like Particles

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The ribonucleocapsids of many plant viruses are extremely stable. The protein coat protects the RNA genome against degradation during the accumulation and spread of progeny virions. Chimeric single-stranded RNA molecules were transcribed in vitro from recombinant plasmids and later encapsidated, in vitro, into ribonucleoprotein particles (pseudoviruses) 60 nanometers long that resembled tobacco mosaic virus. Transcripts encoding an assayable enzyme, chloramphenicol acetyltransferase (CAT), were packaged into pseudovirus particles to assess the utility of this single-stranded RNA delivery system in a wide range of cell types. In all cases, packaged CAT messenger RNA was uncoated and transiently expressed. Significantly higher levels of CAT activity were detected with packaged than with naked CAT messenger RNA after inoculation of plant protoplasts in the presence of polyethylene glycol or abrasive inoculation of intact leaf surfaces. Structural events that lead to the uncoating and expression of CAT messenger RNA showed no cell specificity. This observation may support the view that the comparatively restricted host range of a true plant virus results from events that occur later during the infection cycle.

NUCLEOPROTEIN STRUCTURES OF many viruses, particularly plant viruses, are extremely stable under adverse environmental conditions. The capsid protein protects the genome (DNA or, more especially, RNA) from ubiquitous nucleases during the intracellular accumulation, release, and spread of progeny virions. A number of animal pseudovirus vector systems have been developed (*1–3*) to exploit, in part, this protective function; more importantly, these vector systems increase the efficiency of gene transfer by utilizing the receptor-mediated cell-attachment and uptake properties of animal viruses. Although there is no evidence for such a specific uptake mechanism among plant viruses, we were interested in developing and testing, in vivo, an RNA delivery-expression system based on a well-characterized plant RNA virus, tobacco mosaic virus (TMV) (*4*). The ribonucleocapsids of many plant or animal viruses appear to remain virtually intact as they enter the cytoplasm of the newly infected host cell (*5*). Although this may ensure some continued protection for the messenger-sense viral RNA, it presents a paradox: how is the genome released from such a functionally stable structure under the comparatively mild physico-chemical conditions of the cytoplasm? Some recent evidence suggests that ribosomes may be involved in a process of cotranslational disassembly (*6–8*). To extend our studies on this process in vivo, and to develop a potentially useful protective system for the encapsidation, delivery, and transient expression of single-stranded RNA (ssRNA) molecules in a wide range of cells, we undertook the studies described in this report.

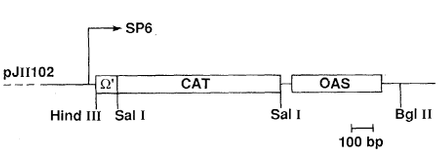

Fig. 1. Portion of the in vitro transcription plasmid pJII102 (*10*) showing the orientation and transcription start point of the bacteriophage SP6 promoter (arrowhead above), the CAT-coding region (0.78 kb), the OAS (0.45 kb) from TMV (U1 strain), and relevant restriction enzyme sites used for insertion into (Hind III and Sal I), or linearization of (Bgl II), the template.

Preliminary work on an SP6-transcript packaging vector has been described elsewhere (*9*). Recently, we reported (*10*) that expression of unencapsidated chloramphenicol acetyltransferase (CAT) messenger RNA (mRNA) is enhanced, both in vitro and in vivo, by the presence of either a 5′-cap structure or a derivative of the 5′-untranslated leader sequence of TMV RNA (Ω′), or both.

Plasmids pJII102 (Fig. 1) and pJII2 (*10*) contained the CAT-coding region from Tn9 (*11*), either with or without the modified 5′-leader sequence (Ω′) from TMV RNA (U1 strain). In vitro transcription of linearized forms of these plasmids resulted in RNA molecules with the TMV (U1) origin-of-assembly sequence (OAS) at the 3′ end. Uncapped or 5′-capped forms of each of these transcripts could be assembled into TMV-like pseudovirus particles (Fig. 2A) 60 nm in length (Fig. 2B), as predicted from the size of the in vitro transcript (1.2 kb) and by comparison with 300-nm-long TMV particles which contain a 6.4-kb genome. By means of ^{32}P-labeled transcripts, it was pos-

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