The Initiation-Promotion-Progression Model of Rat Hepatocarcinogenesis (43511C)

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Abstract. **Carcinogenesis is a multistage process consisting of the three distinct stages: initiation, promotion, and progression. The initiation-promotion-progression (IPP) protocol models these stages and establishes a method whereby agents that possess a carcinogenic risk can be classified as acting primarily at any one or combination of these stages. In one hepatocarcinogenesis IPP protocol, rats were initiated with 10 mg of diethylnitrosamine/kg body wt at 5 days of age, started on the promoting agent phenobarbital at weaning, subjected to a 70% partial hepatectomy at 6 months, and, at the peak of proliferation, given a putative progressor agent, ethylnitrosourea ([ENU] 100 mg/kg, ip) or hydroxy-urea ([HU] 3 × 150 mg/kg, ip). Administration of the promoting agent was discontinued after the progressor agent was given, and the rats were sacrificed 6 months later. The number and volume fraction of promoter-independent (growth in the absence of the promoting agent) altered hepatic foci (AHF) were then determined by quantitative stereology. The number of such AHF increased with either ENU or HU treatment compared with animals not given a progressor agent. In addition, hepatocytes isolated from animals subjected to an IPP regimen with ENU as the progressor agent exhibited a greater degree of chromosomal breakage and aneuploidy than animals not given a second initiator. A variation of this model, in which the promoting agent was maintained after administration of the progressor agent, was examined. In this IPP model, the number of heterogeneous AHF (foci-in-foci) increased after application of the progressor agent (ENU or HU). An increased incidence of hepatocellular carcinoma was also observed in animals subjected to the IPP protocol when promotion was maintained until sacrifice. Thus, the characteristics of progression—increased chromosomal damage, aneuploidy, growth of AHF in the absence of continued tumor promotion, the presence of foci-in-foci, and an increased incidence of malignant neoplasia—have been used as end points for the demonstration of progressor activity by ENU. In addition, the potential progressor activity of HU and benzene has been demonstrated with the IPP model of rat hepatocarcinogenesis.** [P.S.E.B.M. 1993, Vol 202]

Studies of the multistage nature of experimental carcinogenesis have focused primarily on the early, sequential stages of initiation and promotion that result in preneoplastic lesions (1–4). The rat liver model of cancer development has played an important role in our understanding of the processes involved and the changes that accompany preneoplasia (5, 6). The combination of the two-stage model of carcinogenesis, which has proven useful for both the mouse skin (1, 3) and rat liver (2, 4, 7) paradigms, with the concept of progression provided by Foulds (8) has led to a description of the carcinogenic process (9) as occurring in three stages (initiation, promotion, and progression) in both the mouse epidermal (10–14) and rat liver models (6, 15–19).

Progression, as described by Foulds (8), includes all phases of neoplastic development after initiation. The demonstration of an operationally reversible, intermediate stage of promotion that is dependent upon the continued administration of the promoting agent and that does not involve demonstrable changes in the genome (19) suggests that Foulds' concept of progression should be reevaluated. Since promotion, if existent, immediately follows initiation, progression can be considered the terminal stage of neoplastic development (17).

Based on the epidemiology of several human can-

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cers, a description of carcinogenesis by the two-hit model has been forwarded by Moolgavkar, Knudson, and their colleagues (20–23). Additional evidence of the need for a minimum of two genetic changes to evoke malignant neoplasia comes from studies on transformation resulting from cotransfection studies with two activated oncogenes (24). The initiation-promotion-progression (IPP) protocol of chemical carcinogenesis was suggested by Potter (9) and has been used to demonstrate the ability of complete carcinogens (10, 11, 14), selectively cytotoxic agents (12), and free radical generators (13) to induce progression in the mouse skin model. The IPP protocol also models these three stages in the rat liver (18, 19, 25, 26) and, coupled with the technique of quantitative stereology, permits the investigation of each stage separately (19, 25, 26). Several phenotypic markers of altered hepatic foci have been used in two morphologic methods of assessing progressor action.

Early work in the skin model with repeated administration of initiating agents suggested that progressor agents may have characteristics similar to initiating agents (27–30). A similar conclusion was reached later by Hennings *et al.* (10, 11) using the IPP model in which direct-acting mutagens were used as the putative progressor agents. Although classes of chemical agents that present a carcinogenic risk primarily through progressor action have not been established, complete carcinogens, cytotoxic agents, and clastogenic agents are active during this stage of mouse epidermal carcinogenesis (10–14). In addition, urethane has been shown to result in an increased incidence of malignant neoplasms and thus can be considered to be a progressor agent for the skin (29), further suggesting that agents other than direct-acting mutagens are able to begin the progression process.

The complete carcinogen ethylnitrosourea (ENU) has been demonstrated by Scherer (15, 18) and his colleagues (16) to induce the formation of foci-in-foci in the rat liver by use of an IPP format. This morphologic end point may serve as a marker for the identification of progression. In the mouse skin model, progression occurs primarily within preexisting papillomas (14, 29), further suggesting that the foci-in-foci configuration may be a useful end point for progression in the liver. Since one hallmark of advanced malignant lesions is their autonomy from normal growth control and because at least two studies have found only promoter-independent lesions to contribute to the development of carcinomas in the skin (11, 31), another potential method for identification of progressor agents for the liver may be the quantitation of foci that remain after discontinuation of promoting agent administration (i.e., promoter-independent foci). Because numerous investigators have described progression as the stage of evolving karyotypic instability (17, 32–34), any purported model of carcinogenesis should be reconciled with progressive increases in aneuploidy and DNA damage. Therefore, potential end points for the determination, quantitation, and characterization of putative progressor agents in the rat liver should include chromosomal changes, the morphologic parameters of foci-in-foci and promoter-independent foci, and the incidence of malignant neoplasias. The use of such end points to evaluate the initiation-promotion-progression model for the induction of malignant neoplasms in experimental carcinogenesis is described herein.

Methods

Animal Protocols. Male Sprague-Dawley rats were used for the culture experiments, and female rats were used in the morphologic analyses. Concurrent age-matched controls were fed a cereal-based diet. The three formats of animal treatment are provided in Figure 1. The initiation-promotion (IP) format is a modified Peraino procedure (35) in which 5-day-old rats were injected with 10 mg of diethylnitrosamine/kg and promoted at weaning with a diet containing 0.05% phenobarbital for 6 months. In the IPP protocol, the Peraino protocol was combined with a modified Pitot protocol (2). For the foci-in-foci studies, the initiated and promoted animals were subjected to a 70% partial hepatectomy to provide a proliferative stimulus and were then administered the progressor agent. These IPP animals were either maintained on phenobarbital until sacrifice 6 months after progressor administration or were withdrawn from promoter administration after progressor application. In the assessment of promoter-independent foci, the promoter was withdrawn after the second initiation. Both *N*-nitroso-*n*-ethylurea (ENU, 100 mg/kg) and benzene (1 g/kg) were administered at 24 hr after partial hepatectomy, whereas hydroxyurea ([HU] 150 mg/kg) was administered at 20, 30, and 40 hr after partial hepatectomy. More recent evidence (J. Hully, Y. P. Dragan, H. C. Pitot, unpublished observations) suggests that the intrinsic proliferative rate in the focal hepatocytes may provide a sufficient stimulus for progression to ensue and that the partial hepatectomy may be unnecessary.

Morphologic End Points for the Detection of Progressor Agents. Female rats (7–12 per group) treated as described in Figure 1 were sacrificed by decapitation and their livers removed. Sections of each of the three main lobes remaining after partial hepatectomy were arranged as a block and immediately frozen on dry ice. Additional sections were fixed in formalin, processed for routine histologic evaluation, and stained with hematoxylin and eosin. Four sequential sections from each block were stained successively for the placental isozyme of glutathione-S transferase, γ-glutamyltranspeptidase (GGT), canalicular adenosine triphosphatase, and glucose-6-phosphatase by methods

described previously (36). The number of altered hepatic foci (AHF) per liver and the volume percentage of liver containing AHF were determined according to previously published procedures (36–38).

The number of foci-in-foci (Fig. 2) is a qualitative measure of the number of smaller lesions observed within larger lesions (16, 18, 25, 39). This qualitative measure of heterogeneous lesions was obtained by visual inspection of the overlays of the four phenotypic markers used in these studies (25, 39). In this analysis, the nonhomogeneous expression of the four enzyme markers was used to identify smaller (presumably more recent) lesions within larger (presumably older) ones. The number of promoter-independent foci is a stereologically defined parameter of the number of AHF per liver remaining after cessation of promoting agent administration. Routine histologic evaluations were performed on formalin-fixed, paraffin-embedded tissue stained with hematoxylin and eosin.

Isolation, Culture, and Chromosomal Analysis of GGT$^+$ Hepatocytes. A two-step collagenase perfusion procedure was used to prepare a single-cell hepatocyte suspension from five animals per group treated by the IPP protocol described in Figure 1, in which the promoting agent was discontinued after administration of the progressor agent ENU. The isolated cells were subjected to a Percoll purification procedure to remove nonviable hepatocytes and nonparenchymal cells (40).

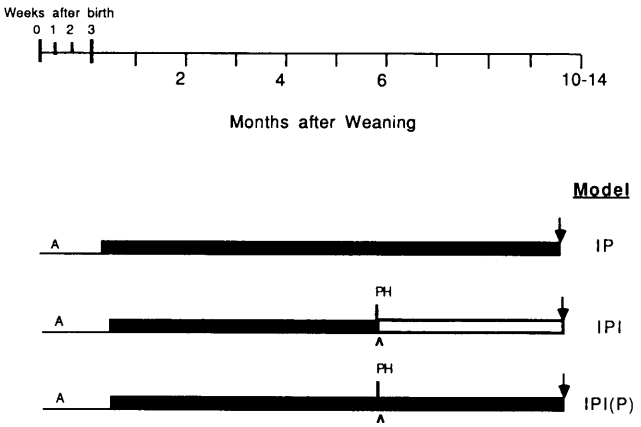

Figure 1. Experimental format for IP and IPP protocols. The IPP model couples the IP model described by Peraino *et al.* (4) with that of Pitot *et al.* (2). Specifically, neonatal rats are initiated at 5 days of age by a single intraperitoneal injection of diethylnitrosamine (10 mg DEN/kg). The rapid growth of the liver at that age provides the proliferative stimulus necessary for initiation. Promotion is begun at weaning, and at 6 months of age a modified Pitot *et al.* (2) protocol is performed in which the rat is given a 70% partial hepatectomy (PH) followed by administration of the putative progressor agent (as described in Methods). The animal is either maintained on phenobarbital for the detection of foci-in-foci or carcinoma incidence (IPI(P)), or administration of the promoting agent is discontinued (IPP) after progressor administration. This latter IPP protocol is used for the detection and quantification of promoter-independent foci. Closed bar, 0.05% phenobarbital in NIH-07 diet; open bar, NIH-07 diet; A, diethylnitrosamine (15 mg/kg); Λ, second initiator given 24 hr after partial hepatectomy; ▼, animals sacrificed.

The procedure of Hanigan and Pitot (41), as modified by Xu *et al.* (42), was followed to allow the separation of GGT$^+$ and GGT$^-$ hepatocytes by a panning technique in which plates coated with anti-GGT antibody were employed to obtain an enriched GGT$^+$ cell population. Next, these two cell populations were plated separately at 2.5×10^5 cells per fibronectin-coated plate. Beginning 48 hr after plating, the hepatocytes were treated for 5 hr with 0.1 μg of Colcemid, detached by collagenase/trypsin treatment, and harvested by treatment with 75 mM KCl. The cells were then fixed with two changes of three parts methanol to one part acetic acid (34). The slides were stained with Giemsa and the number of chromosomes and their breakage patterns were scored. The chromosomes were grouped by size and centromere placement by use of unbanded karyotype analysis, and at least 100 metaphase spreads per animal were assessed (34).

Results

Foci-in-Foci. Morphologically, the earliest detectable lesion after administration of the progressor agent in the IPP protocol may be the "focus-in-focus" as described by Scherer (15, 16, 18) and Pitot (17, 25) and his colleagues (19, 43). The detection of a focus-in-focus (FIF) is dependent upon the growth rate and morphology of the second (internal) focus and is difficult for single phenotypes; however, it may be possible to use the overlays of focal transections obtained for different enzyme markers on serial sections to identify phenotypically distinct, smaller, and presumably more recent lesions (FIF) within larger, presumably older ones (Fig. 2). With this overlay technique, an occasional FIF was observed in diethylnitrosamine-treated animals and in animals treated on an initiation-promotion protocol. Treatment with HU or ENU as putative progressor agents in the IPP protocol resulted in a significant increase in the number of FIF per liver, as well as FIF per AHF (25).

Promoter-Independent Foci. Autonomous growth of AHF or their promoter independence can be determined by employing a modification of the IPP protocol in which administration of the promoting agent is withdrawn after treatment with the putative progressor agent (Fig. 1). The AHF that remain after promoter withdrawal are thus promoter independent and may reflect foci that have sustained a second genetic insult. Treatment of animals on the IPP protocol with a partial hepatectomy 6 months after beginning the promotion regimen, but with subsequent removal of the promoting agent, resulted in a basal level of promoter-independent foci ([PIF] Table I). Table I shows that treatment with either HU or ENU after the partial hepatectomy resulted in a significantly greater number of PIF than observed in animals not receiving a putative progressor agent (19).

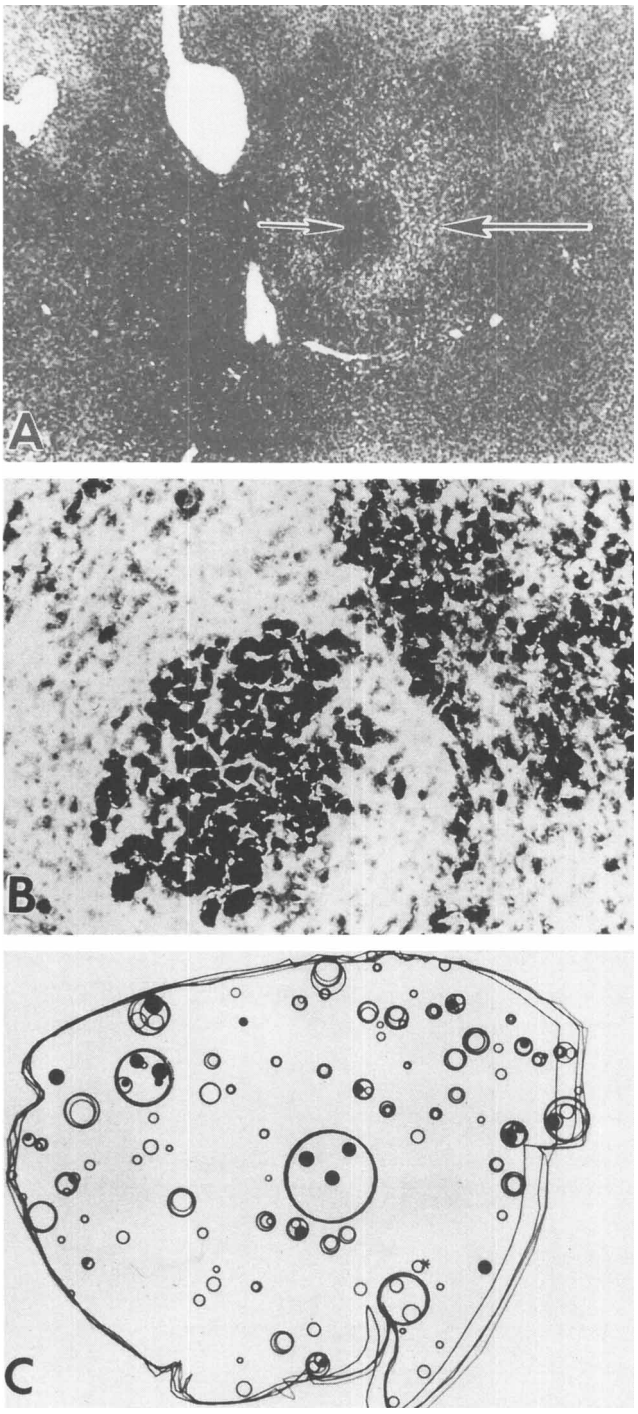

Figure 2. Depiction of the transitional, heterogeneous foci that can be occasionally detected in IP protocols but that are markedly enhanced after administration of a second initiator as in the IPI protocol described by Potter (9). (A) Hematoxylin and eosin-stained liver section demonstrating the presence of a basophilic focus (smaller arrow) within a more eosinophilic nodule (larger arrow). (B) Histochemically stained sections can be used to demonstrate heterogeneous lesions; (B) shows a focus of glucose-6-phosphatase (G6P)-positive cells within a focus of G6P negativity. Overlays of the tracings from serial sections stained for the four different enzymes can be used to qualitatively demonstrate the appearance of (C) focus-in-focus. A quantitative measure of these lesions would be possible only through the use of serial reconstruction.

Table I. The Number of Promoter-Independent Foci Resulting from the Application of Potential Progressor Agents during an IPP Protocol to Female Rats[a]

Treatment (IPI)	No. of AHF/liver (4 markers)
DEN/PB/–	4,900 ± 250
DEN/PB/ENU	18,500[b] ± 1,500
DEN/PB/HU	6,600[b] ± 700

[a] Data are the numbers of promoter-independent (i.e., progressed) AHF per liver determined by quantitative stereology for animals (7–12 female Sprague-Dawley rats) treated on an IPP protocol (see Fig. 1) when ENU or HU was tested as a progressor agent (second initiator). See Methods for further details or protocol.
[b] The observed number of promoter-independent AHF per liver differed significantly from that observed for animals in which no second initiator was administered ($P < 0.05$) (adapted from Pitot *et al.* [19]).

Table II. Comparison of the Percentage of Cells with DNA Damage in GGT^+ and GGT^- Hepatocytes Isolated from Rats Subjected to the IP or IPP Protocol[a]

Protocol	Chromatid	Isochromatid	Fragments	Chromosomal rearrangements
Control	3.0 ± 1.3	0.0	0.0	0.0
IP (Promotion)				
GGT^+	4.0 ± 1.3	0.0	0.0	0.0
GGT^-	2.6 ± 1.0	0.6	0.0	0.0
IPP (Progression)				
GGT^+	28.0 ± 5.0	16.0 ± 6.0	7.0 ± 2.0	17.0 ± 2.0
GGT^-	21.6 ± 2.0	14.0 ± 2.0	16.0 ± 1.0	10.0 ± 5.0

[a] Data show the karyotypic changes observed in age-matched animals treated on the IP or IPP protocol. Details for the procedure are provided in Methods. GGT^+ indicates hepatocytes expressing γ-glutamyltranspeptidase, and GGT^- indicates hepatocytes not expressing this enzyme (as adapted from Pitot *et al.* [19]).

Karyotypic Instability. An important hallmark of progression is an evolving karyotypic instability (32–34) coupled with an increase in the degree of aneuploidy (34, 44). Examination of hepatocytes from animals treated on an IPP format with 100 mg of ENU/kg body wt as the putative progressor agent resulted in a marked increase in chromosome damage, including isochromatid formation, chromosomal rearrangements, and fragmentation, whereas animals treated on the IP or the IPP protocol without administration of a progressor did not differ in chromosomal integrity from untreated animals (Table II). Figure 3 represents the karyotypes of GGT^+ foci that were isolated from animals treated on the IP or IPP protocol. Hepatocytes from untreated rats are predominantly tetraploid, whereas GGT^+ hepatocytes are predominantly diploid (Fig. 3). In contrast, hepatocytes from IPP animals treated with ENU were primarily aneuploid (Fig. 3). Thus, treatment with an agent with progressor action may increase the degree of aneuploidy (43) and chromosomal damage (19) com-

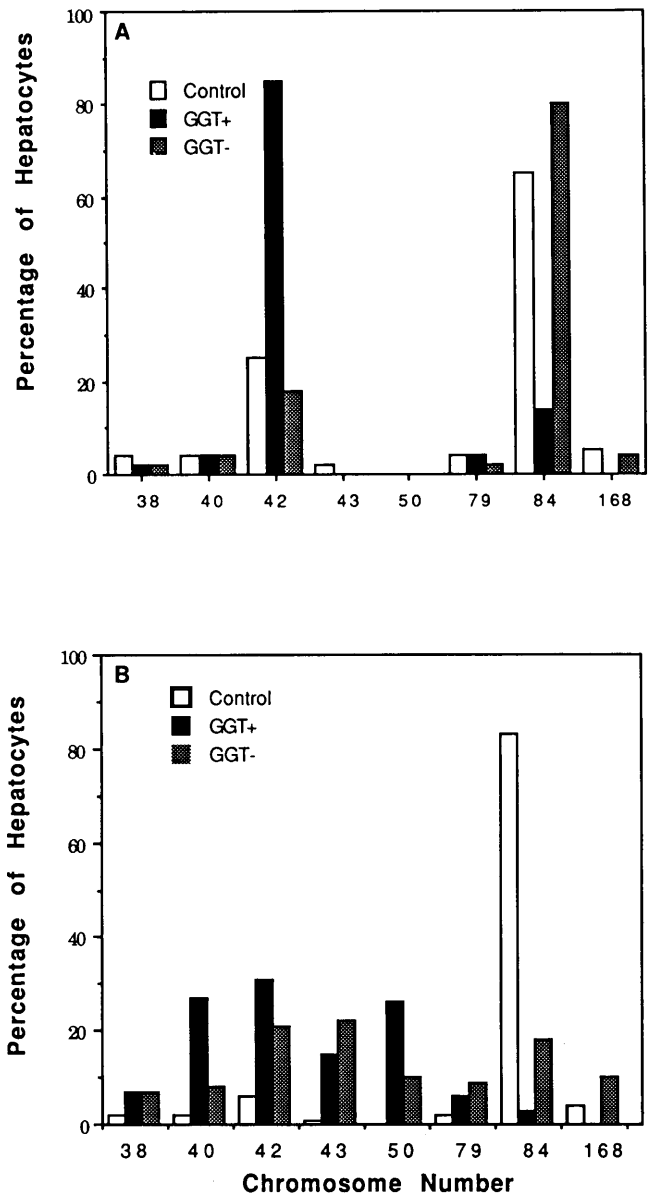

Figure 3. Comparison of the chromosomal ploidy distribution for untreated, promoted (animals subjected to the IP protocol), and progressed (animals subjected to IPP protocol) rat hepatocytes. (A) Distribution of chromosome number for hepatocytes from control rats and for GGT^+ and GGT^- hepatocytes from rats subjected to the IP protocol. (B) Distribution of chromosome number for hepatocytes from control rats and for GGT^+ and GGT^- hepatocytes from rats subjected to the IPP protocol. For details of the experimental protocol see Methods (for chromosomal analyses) (as adapted from Sargent *et al.* [75] and Pitot *et al.* [43]).

pared with parallel-treated animals not receiving the progressor agent (19, 34).

Malignant Tumor Incidence. Perhaps the most definitive characteristic of a progression-inducing agent is an increased incidence of malignant neoplasia compared with animals treated on the same protocol but without progressor administration. In this study, hepatocellular carcinoma incidence per rat was increased with these two agents compared with appropriate control animals (Table III) when the progressor agent was followed by phenobarbital administration. The number of animals with hepatocellular carcinomas in relation to the number per treatment group is provided in Table III. The incidence of hepatocellular carcinoma was low when administration of the promoting agent was discontinued after administration of the progressor agent. In contrast, a marked increase in the incidence of hepatocellular carcinoma was observed after administration of ENU or HU in an IPP protocol compared with appropriate controls when the promoting agent phenobarbital was continued after administration of the progressor agent until sacrifice.

Benzene is a known human carcinogen (45), but its mechanism of carcinogenicity and the stages during cancer development at which it is active are not adequately understood in animal models (45, 46). With the IPP protocol in which the promoting agent is given from weaning until sacrifice, benzene was administered once after a partial hepatectomy to test its action as a progressor agent. Table IV indicates that benzene may have some initiating as well as progressor action at the dose tested; however, stereologic determination of the number of AHF detected with glutathione-S transferase and GGT found very few AHF initiated with benzene and promoted by phenobarbital (Fig. 4), which suggests that benzene is a poor initiator under these conditions. Since a single benzene administration increased the incidence of hepatocellular carcinoma in both male and female rats compared with appropriate controls, benzene may possess progressor activity and increase the risk of cancer development through action at this stage of carcinogenesis.

Discussion

The two morphologic end points, foci-in-foci and promoter-independent foci, which were tested for their ability to allow the detection and quantitation of the

Table III. Hepatocellular Carcinoma Incidence in Female Rats Treated on an IPP Protocol[a]

Treatment	Incidence
IPI	
DEN/PB/-/-	3/14
DEN/PB/HU/-	1/12
DEN/PB/ENU/-	2/17
IPI(P)	
DEN/PB/-/PB	1/8
DEN/PB/HU/PB	6/9
DEN/PB/ENU/PB	8/9

[a] The incidence of hepatocellular carcinoma formation was scored as a function of the number of animals in that treatment group. Dosages and abbreviations used in table: DEN, 10 mg of diethylnitrosamine/kg body wt; PB, 0.05% phenobarbital admixed in the diet; HU, 3 × 150 mg of hydroxyurea/kg body wt; ENU, 100 mg of ethylnitrosourea/kg body wt. The criteria of Squire and Levitt (77) were used for the histologic diagnoses.

Table IV. Progression by Benzene in Rat Hepatocarcinogenesis

Treatment	AHF	Hepatic nodules	Hepato-cellular carcinoma
Male rats			
Benzene	6/6	0/6	0/6
DEN/benzene	7/7	7/7	0/7
Benzene/PB	8/9	4/9	0/9
DEN/PB	24/24	24/24	1/24
DEN/PB/benzene	7/7	7/7	5/7
Female rats			
Benzene	8/9	1/9	0/9
DEN/benzene	11/11	11/11	0/11
Benzene/PB	14/15	11/15	0/15
DEN/PB	19/19	19/19	8/19
DEN/PB/benzene	9/9	9/9	8/9

[a] Liver histopathology in rats treated on an IPP protocol (Fig. 1) in which phenobarbital treatment was maintained after the administration of benzene, a putative progressor agent. Dosages and abbreviations used in table: DEN, 10 mg of diethylnitrosamine/kg; PB, 0.05% phenobarbital; benzene, 1 g/kg administered per os. The criteria of Squire and Levitt (77) were used for the histologic diagnoses.

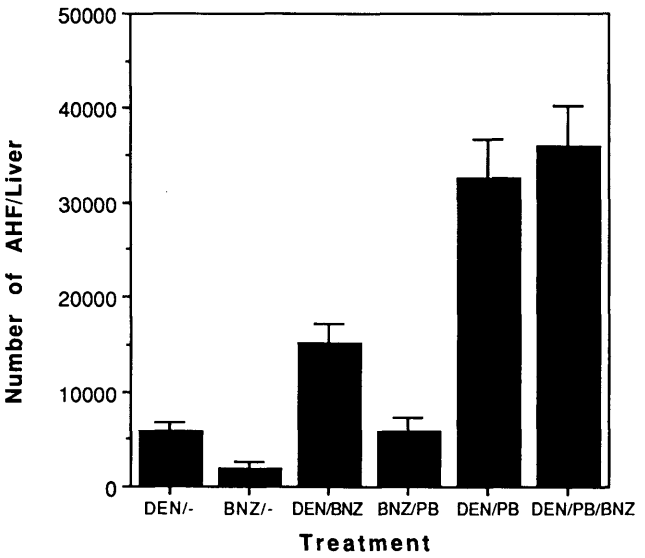

Figure 4. The number of altered hepatic foci in male rat liver of animals treated on an IPP protocol in which benzene was administered once 24 hr after a 70% partial hepatectomy. DEN indicates initiation at 5 days of age with 10 mg of diethylnitrosamine/kg; PB indicates promotion with 0.05% phenobarbital as the free acid admixed into the diet; and BNZ indicates 1 g of benzene/kg administered by gavage.

stage of progression, were significantly elevated after the administration of a complete carcinogen (ENU) or a clastogenic agent (HU) as the progressor agent in an IPP model of hepatocarcinogenesis. The FIF parameter is qualitative and not a true reflection of all FIF in the liver, but rather a morphologic marker of the progressive clonal development of initiated cells toward malignant neoplasms (11, 14, 18). The increase in AHF per liver with ENU treatment, but not with HU treatment, indicates that ENU, but not HU, has initiating action in the rat liver under these conditions. Both agents increased the FIF per liver and the FIF per AHF parameters, indicating, however, that they may both act as progressor agents. The increase in the FIF parameter suggests but does not prove the complete evolution of a single cell to a malignant neoplasm in the rat liver (18). Studies on experimental carcinogenesis in colon (47), liver (18, 48), intestine (49), glandular stomach (50), esophagus (51), urinary bladder (52), respiratory epithelium (53), and mammary cancer (54–58) suggest that this phenomenon is common to many tumor types. In addition, epidemiology (20–23) and examination of human tissue (59) indicate the potential relevance of FIF and the progression concept to human cancer development (59).

Since a primary characteristic of malignant growth is autonomy, perhaps those lesions that grow in the absence of a promoting agent are most likely to progress to carcinoma. In the mouse skin model, promoter-independent but not promoter-dependent papillomas result in carcinomas (11, 31). Hepatocytes isolated from AHF of initiated and promoted animals when transplanted into syngeneic hosts are promoter dependent (60). The application of a progression-inducing treatment may provide the stimulus necessary to propel a benign tumor toward frank malignancy. This inducement, coupled with the removal of the promoting agent, should then result in some foci that continue to grow in the absence of a promoting agent (i.e., that are promoter independent). The work of Rotstein and Slaga (12) has further suggested that progression in the mouse skin model is not affected by withdrawal of the promoting agent; by analogy, promoter-independent foci in the liver model may represent focal hepatic lesions in the stage of progression. Since the number of promoter-independent foci is a quantitative indication of progression, it can be used to rank by potency putative progressor agents and processes. Both ENU and HU induce a significantly greater number of PIF than observed in appropriate control rats. In this analysis, ENU is a more potent progressor agent in rat liver than HU (26).

Changes in the genome (61–64), and more specifically an abnormality in the number and structure of chromosomes, have been described as primary characteristics of cancer cells (63). Several studies have shown an early diploidization of nodules and hepatocellular carcinomas (44, 64, 65). Experimental carcinogenesis has also assigned a diploid character to early lesions (34, 66–72). These early preneoplastic changes to diploidy, with later progression to aneuploidy found in skin (33) and liver (34), bear upon earlier work by Becker *et al.* (44) that found that nodules had single cells that were aneuploid, but carcinomas were largely aneuploid. In addition, numerous chromosomal abnor-

malities have been observed in hepatocellular carcinomas (72–75), thereby supporting the contention that accumulation of genetic alteration is characteristic of the stage of progression.

Hepatocytes isolated from altered hepatic foci of animals treated on an IPP protocol exhibit aneuploidy and marked chromosome damage compared with those on an IP protocol. Specifically, promotion with phenobarbital resulted in an increase in the percentage of diploid cells without an increase in chromosomal damage above that observed in age-matched controls. Progression (with ENU as the progressor agent), however, resulted in a subtetraploid population with a marked increase in chromosome breakage, translocations, and aneuploidy when compared with hepatocytes from animals that had not been administered a progressor agent. These data are consistent with the hypothesis that the promoted cell population is the preneoplastic population in the liver and support the suggestions of Moolgavkar, Knudson, and their colleagues (20–23) for the appropriateness of the multistage model for carcinogenesis. Epidemiology in humans (20, 59, 76) also supports a two-hit process as important in the development of at least some human cancers. Thus, the IPP format of hepatocarcinogenesis, as first suggested by Potter (9), results in increases in chromosomal damage and changes in ploidy consistent with progressive changes toward a frankly neoplastic state.

The standard IP protocol in the rat liver produces hepatocellular carcinomas at a very low frequency, which is increased in animals subjected to an IPP protocol when progression induced by either ENU or HU is followed by an additional period of promotion. The IPP protocol of Potter results in an experimental regimen that mimics the natural history of cancer development in the human and that may be used to assess the factors responsible for neoplastic growth. In addition, the IPP model allows the classification of agents as acting at any one or all of these three stages of carcinogenesis. Specifically, the data discussed in this paper suggest that some agents possess a carcinogenic potential through their action at the stage of progression and that these agents can increase the likelihood of malignant carcinoma development. The data herein further suggest that two genetic insults create the stage for cancer development if a proliferative stimulus is maintained.

Progression is thus a dynamic evolving process that results in frankly malignant neoplastic growth and that differs from promotion in that a more autonomous class of lesions can be distinguished within the putative preneoplastic ones. The AHF resulting from IPP treatment compared with IP treatment may be more autonomous in growth, since many of these AHF are promoter independent. In addition, hepatocytes in the stage of progression show evidence of karyotypic instability and, in the presence of continued phenobarbital administration, a higher incidence of hepatocellular carcinoma. These results suggest that two genetic insults coupled with changes in normal growth control can result in liver cancer development and that administration of a promoter can influence the growth of hepatocytes in the stage of progression. In summary, Figure 1 shows the two IPP models tested in this laboratory for the detection of putative progressor agents in the rat liver. The middle scheme presents the model suggested by Potter (9) and later tested by Scherer (15, 18) and his colleagues (16). This model allows the determination of PIF, but did not result in an increased incidence of carcinomas with ENU or HU as the progressor agent. The lower scheme, in which promotion is continued from initiation until sacrifice, permits detection of foci-in-foci, as well as of an increased carcinoma incidence. Thus, specific characteristics of progression (Table V) can be employed as end points for the identification of putative progressor agents with the IPP protocol.

Table V. Suggested Characteristics of Progressor Agents in Rat Liver that Can Be Used as Potential End Points for the Identification of Putative Progressor Agents

1. Karyotypic instability
2. Foci-in-foci
3. Promoter-independent foci
4. Tumor incidence

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