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Quantitative Comparison of Initiation and Mutation Phenotypes in Hepatocytes of the Analbuminemic Rat

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The potential relationship between mutagenesis and carcinogenesis has been examined in the Nagase analbuminemic rat treated with a single dose of benzo[*a*]pyrene, an incomplete liver carcinogen. The apparent mutation rate at the albumin locus was calculated by determining the number of hepatocytes expressing a cross-reactive product of albumin in analbuminemic rats treated with benzo[*a*]pyrene. The rate of initiation, the first stage in carcinogenesis, was determined by assessing the number of hepatocytes expressing the placental isozyme of glutathione *S*-transferase (PGST) after administration of benzo[*a*]pyrene. Since the expression of PGST may represent hepatocellular changes independent of initiation, promotion with phenobarbital was employed to clonally expand those putatively initiated hepatocytes expressing PGST. With immunohistochemical measures to assess changes in albumin expression, a threefold increase in the number of hepatocytes expressing albumin was detected after administration of benzo[*a*]pyrene in Nagase analbuminemic rats. A more than fivefold increase in altered hepatic foci (AHF) exhibiting increased PGST expression was observed in animals given benzo[*a*]pyrene treatment followed by phenobarbital, compared with those given benzo[*a*]pyrene alone. The number of albumin-expressing single hepatocytes detected was of the same order of magnitude as the number of individual hepatocytes and AHF expressing PGST, suggesting that similar events may be involved in their formation. Since 3×10^6 single hepatocytes expressing albumin were found in the analbuminemic rat liver after a single administration of benzo[*a*]pyrene, while less than 2×10^4 AHF expressing PGST were observed, formation of individual hepatocytes expressing albumin was a far more frequent event than clonal expansion of initiated hepatocytes in the Nagase analbuminemic rat. However, the number of loci of PGST expression including AHF and single hepatocytes is comparable to that of single hepatocytes expressing albumin.

Key words: Hepatocarcinogenesis — Initiation — Mutation — Analbuminemic rat — Glutathione *S*-transferase

Carcinogenesis is a multistage process, as demonstrated by experimental studies in several organ systems of multiple animal species and by epidemiologic studies of human cancer development.[1,2] In multistage hepatocarcinogenesis, the sequential stages of initiation, promotion, and progression have been defined and characterized.[2-4] The first stage of carcinogenesis, initiation, is accompanied by permanent alterations in the genetic material of the cell; these render the initiated cell more susceptible to neoplastic development than uninitiated cells.[5] In the presence of a promoting agent, some initiated hepatocytes exhibit enhanced proliferation and form colonies of altered hepatic foci (AHF) that aberrantly express specific protein markers.[6-10] The placental isozyme of glutathione *S*-transferase (PGST) is one of the most effective markers that score AHF.[11-14] Recently, single PGST-positive hepatocytes have been observed in untreated aged rats[15,16] and in rats administered a mutagen.[17-19] Since colonies of PGST-positive hepatocytes develop in these animals when a promoting agent is administered,[20] single hepatocytes expressing PGST have been considered to be initiated cells. Since AHF develop from single initiated hepatocytes,[21] the total number of single PGST-positive cells and AHF expressing PGST may compose at least one population of hepatocytes with increased susceptibility for neoplastic development.

Animal models have proven a useful tool in biomedical research of human disease. The Nagase analbuminemic rat is sensitive to carcinogens at several organ sites including the liver.[22] This mutant is a homozygous recessive for the loss of albumin expression owing to a 7-base-pair deletion in the albumin gene.[23-25] Since albumin expression is restored in some hepatocytes after mutagen administration,[23,26] hepatocytes with apparent reverse mutations at the albumin locus leading to reexpression of albumin can be quantified in the liver of these rats.

Benzo[*a*]pyrene (B[*a*]P) administration, when coupled with a proliferative stimulus and subsequent administration of a promoting agent, induces AHF, hyperplastic nodules, and carcinomas in the rat liver.[27-31] In addition,

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its polycyclic structure results in bulky DNA adducts,[32-34] and formation of this type of adduct is accompanied by a restoration of albumin expression in analbuminemic hepatocytes exposed to other mutagens.[23,26] Since B[*a*]P is an incomplete carcinogen in rat liver (one capable of inducing initiation but not complete carcinogenesis),[2,35,36] this compound was used as an initiating agent in a two-stage model of hepatocarcinogenesis in the analbuminemic rat. In this study, initiation quantitated by the appearance of PGST-positive hepatocytes and AHF was compared with the reversion to albumin production of single or small groups of hepatocytes in the Nagase analbuminemic rat administered a single dose of B[*a*]P.

MATERIALS AND METHODS

Thirty analbuminemic rats (obtained from the Sasaki Institute, Tokyo, and bred as a colony at the McArdle Laboratory, Madison, Wisconsin) were divided into three treatment groups, each containing five males and five females. Three days after birth, animals in groups 1 and 2 were given a single intraperitoneal injection of B[*a*]P (Calbiochem, San Diego, CA) at a dose of 300 mg/kg body weight, suspended in 0.1 ml of corn oil.[36] From 21 days of age, animals in group 1 were fed NIH-07 diet (Zeigler Brothers, Gardners, PA), while animals in group 2 were fed NIH-07 diet containing 0.05% phenobarbital (PB). Animals in group 3 (vehicle control) were injected with corn oil and placed on the NIH-07 diet without PB. All animals were killed by decapitation at 15 weeks of age.

The liver was fixed *in situ* by perfusion with phosphate-buffered saline (PBS) followed by perfusion with 10% formalin in PBS. The liver was excised and cut into 2-mm sections, which were dehydrated and embedded in paraffin. Serial sections were cut at a thickness of 5 μm. Sections were incubated at 37°C for 30 min and then deparaffinized by xylene-ethanol treatment.

Sections to be stained for albumin were first treated with avidin (Zymed, San Francisco, CA) for 10 min to block endogenous biotin, followed by 5% normal rabbit serum (Zymed) for 60 min. Sections were then incubated with an affinity-purified, goat anti-rat albumin antibody. Antibody specificity was confirmed by Western blot analysis of chromatographically pure rat albumin (Cappel, Durham, NC) and with liver cytosol from a normal Sprague-Dawley rat. Biotinylated rabbit anti-goat IgG antibody (Sigma, St. Louis, MO) was added, and 60 min later StrepAvidin linked to horseradish peroxidase (Zymed) was added for 10 min. Sections were stained with 3 - amino - 9 - ethylcarbazole (AEC) chromagen (Zymed) for 10 min and counter-stained with Mayer's hematoxylin solution.

The liver sections were stained for PGST by the avidin-biotin-complex method (Vector, Burlingame, CA). Sections were treated with rabbit anti-rat-PGST antibody for 30 min, biotinylated goat anti-rabbit-IgG antibody for 30 min, and StrepAvidin linked to horseradish peroxidase for 30 min. Sections were then stained with AEC.

The number of albumin-positive hepatocytes (Fig. 1A) was determined by examining 4000 hepatocytes from each animal (1000 cells from each of four different sections). The number of albumin-positive hepatocytes per 4000 cells was adjusted to the number of albumin-positive cells per liver by using the total number of hepatocytes in the liver of a 15-week-old rat as determined by Laird and Barton.[37] Fields to be counted were randomly chosen and counted at 400× magnification. Occasionally, a focus of albumin-positive hepatocytes was encountered (Fig. 1B). PGST-positive AHF were enumerated by the method of quantitative stereology as outlined by Campbell *et al.*[38]

In order to score the PGST-positive single hepatocytes and those focal lesions too small to be quantitated accurately by Campbell's method (<10 cells), the PGST-stained sections were also scored and quantitated in the same manner as the albumin-stained sections (Fig. 1C). The number of single PGST-positive hepatocytes or small PGST foci obtained by this method was added to the number of PGST foci (Fig. 1D) detected by the method of Campbell *et al.*[38] to provide a more accurate measurement of the total number of putatively initiated cells. Statistical significance of differences between groups was determined by Mann-Whitney nonparametric analyses.

RESULTS

The number of PGST-positive colonies calculated by the method of Campbell *et al.*[38] is depicted in Fig. 2. Colonies of AHF were not detected in vehicle control-treated analbuminemic rats. Some AHF were detected in male and female analbuminemic rats that received the incomplete carcinogen B[*a*]P as an initiating agent, but that were not treated with PB. These AHF probably resulted from endogenous promotion.[39] The number of AHF per liver in rats receiving only B[*a*]P did not differ significantly between the sexes, with 1870 ± 510 observed in males and 1010 ± 660 observed in females. Analbumenic rats that had received both the initiating and the promoting agent had a significantly greater number of AHF per liver than those which had received only the initiating agent ($P\leq0.05$). No significant difference between male and female rats in the number of AHF per liver was observed following initiation with B[*a*]P and promotion with PB. In male analbuminemic

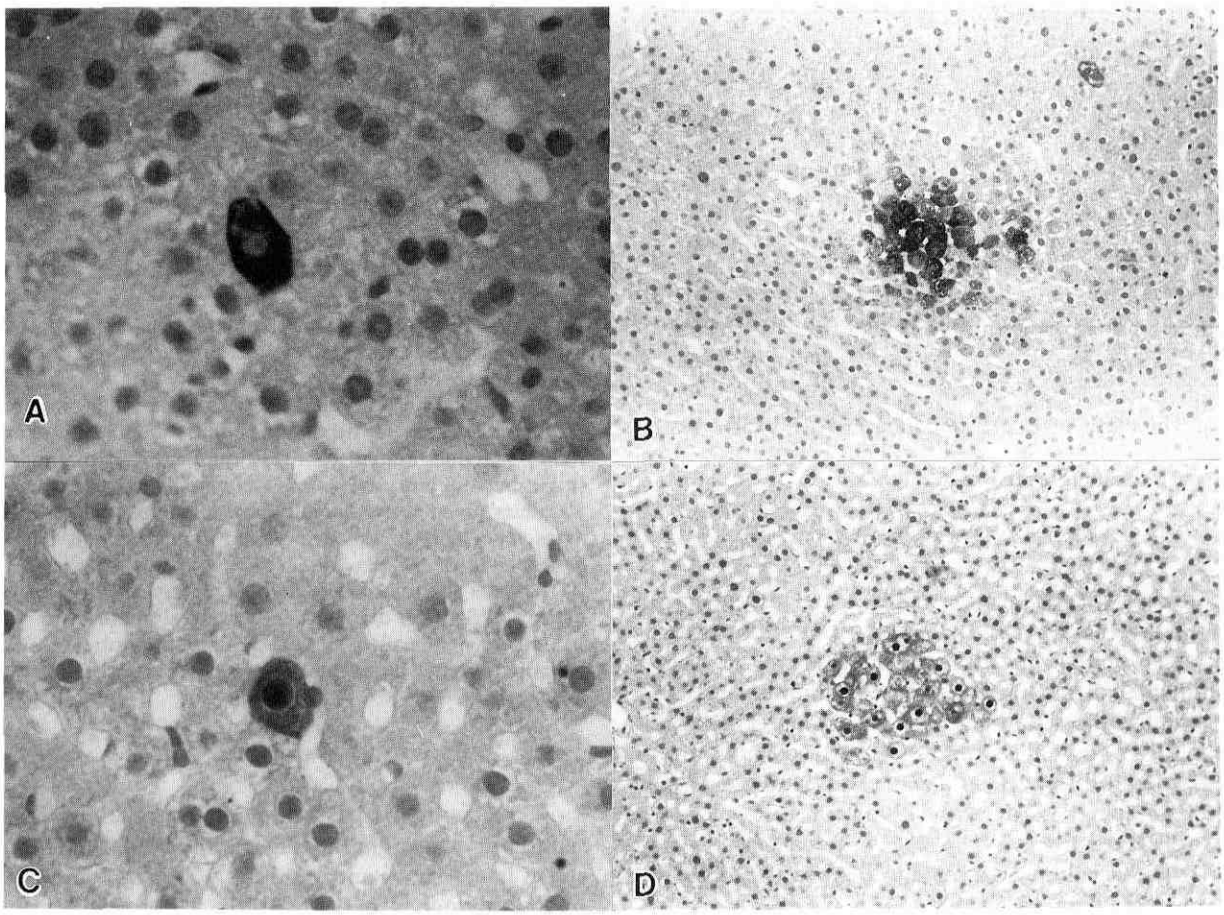

Fig. 1. Photomicrographs of Nagase analbuminemic rat liver depicting A) a single hepatocyte expressing albumin, B) a focus of hepatocytes expressing albumin, C) a single PGST-positive hepatocyte, and D) a focus of hepatocytes expressing PGST.

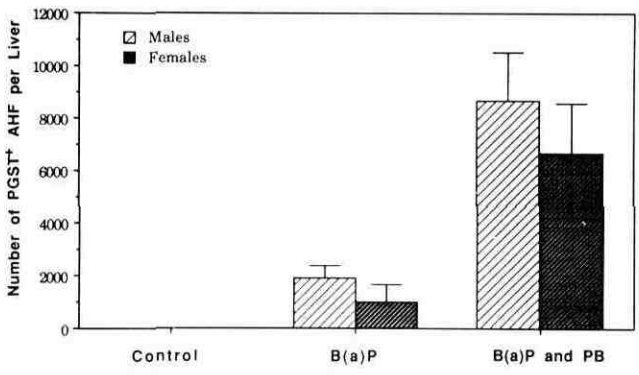

Fig. 2. The effect of B(*a*)P initiation and PB promotion on the number of altered hepatic foci per liver in analbuminemic rats. The number of altered hepatic foci greater than 125 μm in diameter in the liver of male and female analbuminemic rats initiated with 300 mg B(*a*)P/kg body weight was calculated according to the method of quantitative stereology.

rats, the number of AHF per liver was calculated to be 8660 ± 1840, while 6680 ± 1900 AHF per liver were found in the female analbuminemic rat. If the data are combined across sexes, then the average number of AHF per liver in B[*a*]P-treated animals without PB was 1540 ± 400, while that of B[*a*]P animals administered PB was more than five times greater, 8260 ± 1440. Thus, hepatocarcinogenesis in the analbuminemic rat appears to be a multistage process when B[*a*]P, an incomplete liver carcinogen, and PB, a promoting agent, are administered sequentially.

The volume percentage of the liver occupied by AHF is a more direct measure of the growth potential of AHF than the number of AHF per liver, since the volume of an AHF reflects the number of altered cells composing that altered focus. Figure 3 shows the results of measurement of the volume percentage of the liver occupied by PGST-positive foci in analbuminemic rats treated with vehicle alone, B[*a*]P, or B[*a*]P plus PB. AHF were not observed

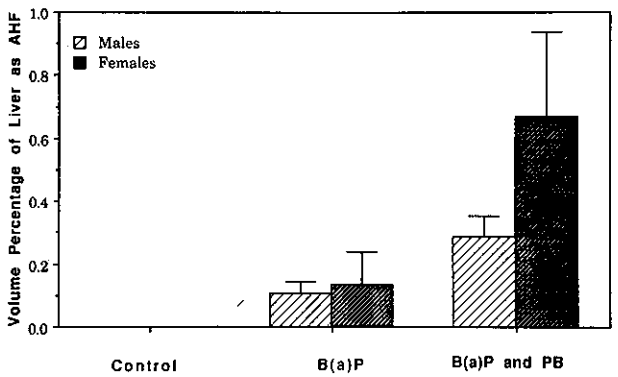

Fig. 3. The effect of B(*a*)P initiation and PB promotion on the percentage of liver occupied by PGST-positive altered hepatic foci in analbuminemic rats.

in the vehicle-treated group. While a concurrent group receiving only PB was not included in this study, unpublished observations (Y. Dragan, J. Peterson, H. Pitot) have indicated a negligible effect on albumin or PGST expression in otherwise untreated NAR rats. The largest PGST-positive colonies were observed in the group treated with both B[*a*]P and PB. This increase in size of B[*a*]P-induced AHF was significant in both sexes following PB treatment ($P \leq 0.05$). In female rats treated with B[*a*]P, a volume percentage of 0.11 ± 0.03 of the liver was occupied by AHF, while in male rats this parameter was 0.13 ± 0.01. When PB was administered after B[*a*]P initiation, a volume percentage of 0.29 ± 0.07 and 0.67 ± 0.27 was observed in male and female analbuminemic rats, respectively. Combining the data on volume percentage for the sexes results in an average volume percentage of 0.12 ± 0.04 for B[*a*]P treatment and 0.37 ± 0.08 for B[*a*]P followed by PB.

The calculated number of AHF resulting from the quantitative stereological calculation of the number of AHF per liver does not include the single hepatocytes expressing PGST and the PGST-positive foci below the detection limit of 125 μm. Therefore, calculation of the number of AHF per liver may underestimate the number of putatively initiated hepatocytes. The total number of PGST-positive loci per liver, including single cells and foci (of fewer than 10 hepatocytes), is presented in Table I. No significant difference was observed between males and females ($P \leq 0.05$); therefore, the data were averaged without regard to gender. The average number of single hepatocytes plus foci less than 125 μm expressing PGST per 4000 cells was lower in the B[*a*]P-treated group than in the B[*a*]P-treated group with PB promotion. A 30-fold greater number of PGST-positive loci were observed in the B[*a*]P-treated rats with PB promotion than in those receiving B[*a*]P but no PB and killed at corresponding times. The average number of PGST-positive single hepatocytes and small (< 125 μm) foci in B[*a*]P-treated animals was $0.45 \pm 0.03 \times 10^6$, while $22.5 \pm 1.4 \times 10^6$ were found in B[*a*]P plus PB-treated animals. PGST-positive hepatocytes were observed only rarely in the vehicle-treated group and did not occur in the randomly chosen fields scored.

Table I. The Effect of Treatment with a Single Dose of 300 mg Benzo[*a*]pyrene/kg Followed or not by Phenobarbital Promotion on the Number of Single Hepatocytes Expressing the Placental Isozyme of Glutathione *S*-transferase (PGST)[a)]

Treatment	Single PGST^{+}[b)] hepatocytes/4000 cells	Single PGST^{+} hepatocytes/liver
Control	–	–
Benzo[*a*]pyrene	0.86 ± 0.65	0.45×10^6
Benzo[*a*]pyrene + Phenobarbital	27.0 ± 10.5[c)]	22.5×10^6

a) Single hepatocytes and foci of hepatocytes < 125 μm expressing PGST were determined on paraffin sections stained immunohistochemically for PGST by counting the number per 4000 in randomly chosen fields expressing this marker. The frequency of single hepatocytes and small foci expressing PGST was determined from this number. The total number of single hepatocytes and small foci per liver expressing PGST was determined by extrapolation based on the number of hepatocytes reportedly in the liver of an animal of this age with a liver weight of the size observed.
b) Reflects the total of single hepatocytes and foci of hepatocytes < 125 μm in diameter expressing albumin.
c) Differs significantly ($P < 0.05$) from the group treated with benzo[*a*]pyrene alone with Mann-Whitney nonparametric analysis.

As shown in Table II, the number of albumin-positive hepatocytes per liver in the B[*a*]P-treated group (3.3×10^6) was three times greater than in the controls (1.1×10^6). The calculated number of albumin-positive hepatocytes did not differ significantly ($P < 0.05$) between the sexes, and the numbers were therefore averaged. There were no detectable albumin-positive hepatocytes in the livers of analbuminemic rats treated with both B[*a*]P and PB. This result may be explained by the finding of Kasper and his associates[40] that PB inhibits the expression of albumin in rat hepatocytes.

DISCUSSION

The threefold increase in albumin-positive hepatocytes after administration of the incomplete carcinogen B[*a*]P indicates that this polycyclic aromatic hydrocarbon increases the reverse mutation rate in the analbuminemic rats at the albumin locus. This increase is lower than, but in fairly close agreement with results obtained by

Table II. The Effect of Treatment with a Single Dose of 300 mg Benzo[*a*]pyrene/kg in the Absence of Phenobarbital Promotion on the Number of Single Hepatocytes in the Nagase Analbuminemic Rat Expressing Albumin[a)]

Treatment	Single albumin$^+$ hepatocytes/4000 cells	Single albumin$^+$ hepatocytes/liver
Control	2.2 ± 0.3	1.11×10^6
Benzo[*a*]pyrene	6.2 ± 0.9[b)]	3.31×10^6

a) Single hepatocytes expressing albumin were determined on paraffin sections stained immunohistochemically by counting the number per 4000 in randomly chosen fields expressing this marker. The reversion frequency was determined from this number. The number of single cells per liver expressing albumin was determined by extrapolation based on the number of hepatocytes reportedly present in the liver of an animal of this age with a liver weight of the size observed.
b) Differs significantly ($P<0.05$) from the group treated with benzo[*a*]pyrene alone with Mann-Whitney nonparametric analysis.

several other groups who demonstrated the appearance of albumin-positive hepatocytes in analbuminemic rats after treatment with 3′-methyl-4-dimethylaminoazobenzene or 2-acetylaminofluorene.[23,26)] These compounds form bulky adducts in DNA and have an ability similar to B[*a*]P to induce albumin-positive cells in the analbuminemic rat liver.

The induction of PGST-positive AHF in the analbuminemic rat liver following a carcinogenic dose of the complete carcinogen diethylnitrosamine has been reported.[41)] Nodules positive for PGST have been observed in a second similar study.[42)] In addition, Cameron[19)] has reported the appearance of single PGST-positive hepatocytes in normal rats after administration of B[*a*]P. The total number of albumin-positive hepatocytes following B[*a*]P administration without PB is approximately six times greater than the number of single PGST-positive hepatocytes and small foci (<125 μm) under the same conditions. The magnitude of the number of hepatocytes that can be detected by the reestablishment of albumin expression and the aberrant expression of PGST suggest that the reverse mutation rate at the albumin locus may be greater than the rate of initiation of PGST-positive hepatocytes and their clones.

Albumin-positive cells could not be detected in animals treated with PB. Several studies have demonstrated a post-transcriptional modulation of albumin mRNA upon administration of PB,[40,43)] and this may explain the decreased albumin expression observed. Since the animals treated with B[*a*]P and PB received the same dose of B[*a*]P as the animals treated with B[*a*]P alone, one may assume that as many hepatocytes with mutations existed in the group treated with B[*a*]P and PB as in the group treated with B[*a*]P alone on the basis that PB is nonmutagenic.[44)]

The larger number of PGST-positive single hepatocytes, small foci, and AHF per liver in the B[*a*]P plus PB group compared with the group receiving only B[*a*]P was owing primarily to the large number of single or double hepatocytes expressing PGST in the group treated with PB. Although recent evidence suggests that this may partially be an age-related effect of a commercial diet,[20)] a large number of small PGST-positive foci (<125 μm) was observed only in the B[*a*]P plus PB-treated group. The increased number of PGST single cells, small foci, and AHF in the presence of PB suggests that this promoting agent increases the expression of PGST in addition to enhancing the growth of hepatocytes that express PGST. However, PB inhibits apoptosis of single PGST-positive hepatocytes, as noted by Bursch *et al.*[45)] Therefore, it is likely that PB induces the expression, but not the proliferation, of the PGST-positive single and double hepatocytes. Since relatively few single PGST-positive cells have been observed in the livers of the animals that received only PB (data not shown), the large number of small PGST-positive foci (<125 μm) observed in animals treated with B[*a*]P and PB is not the result of induction of PGST-positive hepatocytes by PB administration. Instead, their presence may result from an increase in proliferation of hepatocytes induced by PB, as has been suggested to explain the effect of a partial hepatectomy on albumin expression.[26)] Since the number of PGST-positive lesions in the presence of PB is much greater than the number of albumin-positive hepatocytes in its absence, PB administration may result in more than growth of the initiated cell population and thus may alter phenotypic expression, decrease cell turnover, or increase cellular proliferation of hepatocytes that have been exposed to a mutagenic or initiating event.[42)] Alternatively, there may be a loss of hepatocytes expressing PGST when a promoting stimulus such as PB is not provided.[18)] Comparisons of phenotypic changes at the albumin and PGST loci assume that the efficiency of detection of cross-reactive material at each locus by polyclonal antibodies is similar, and in addition that all mutations at each locus result in a quantitatively similar level of detection of altered cells by a change in phenotypic expression. Although further work is necessary to characterize whether specific mutations lead to initiation, the analbuminemic rat may serve as a useful model for a simultaneous *in vivo* assessment of the mutagenic and initiating potential of carcinogenic agents such as the aryl hydrocarbon benzo[*a*]pyrene.

The apparent high rate of reversion to phenotypic expression of albumin in the analbuminemic rat induced by administration of B[*a*]P *in vivo* compared with that

observed for different loci in *in vitro* mutagenesis experiments[46-55] may indicate a limitation of this model for mutagenesis assays. However, the observed high background rate (spontaneous reversion rate) of albumin expression[23,26] indicates that the splicing deficit responsible for the lack of serum albumin in analbuminemic rats may be overcome by aging or mutagen administration.[26,56] Since the albumin gene in the analbuminemic rat is transcribed but not processed properly owing to a deletion in the 5′ splicing junction,[24] further mutation of the albumin gene is presumably responsible for reestablishment of albumin expression. The 5′ end of introns is important for proper splicing,[57-59] and alteration of the 5′ consensus sequence of a number of genes is known to alter the pattern of expression of their products.[60-67] In analbuminemic rats, a part of the 5′ consensus sequence that is not normally well conserved is deleted and appears necessary for splicing.[26,56,68] Immunocytochemistry and *in situ* hybridization have shown that only a few hepatocytes express albumin in the analbuminemic rat.[23,26,56,69] Truncated albumin proteins have been demonstrated in analbuminemic rats,[56,70] suggesting that, while the splicing defect can be overcome, an abnormal but cross-reactive (to polyclonal albumin antibody) protein is produced. The consensus sequences of splice sites have been proposed as hot spots for mutation.[71] In addition, B[*a*]P has been shown to induce splice site mutants, as well as reversions of such mutants at the *dhfr* locus.[71,72] Thus, the high rate of reversion to expression of albumin in NAR induced by B[*a*]P may be due to mutation. The analbuminemic rat therefore may provide a useful *in vivo* model in which to assess the importance of the 5′ consensus sequence in splicing and the role of chemically mediated mutagenesis.

The prevalence of $PGST^+$ AHF formation per hepatocyte in this study is of the same order of magnitude as that described for different AHF markers by Emmelot and Scherer[73,74] and Farber.[75] Using the loss of canalicular ATPase as a marker of focal change, Emmelot and Scherer[73,74] have calculated that administration of the same molar dose of diethylnitrosamine (DEN) as B[*a*]P used in this study resulted in $0.2–1\times10^4$ AHF per liver. This corresponds to approximately 1 AHF formed per 10^5 hepatocytes with this dose of DEN. A similar number of GGT^+ AHF per liver was determined by Farber[75] to result from 200 mg DEN/kg in the resistant hepatocyte model. The present data indicate that 9×10^4 $PGST^+$ AHF can be detected following initiation with 300 mg/kg B[*a*]P and promotion with PB. The number of single hepatocytes and small foci $< 125\ \mu m$ exhibiting increased PGST expression is at least 2 orders of magnitude higher than the number of $PGST^+$ AHF. These data indicate that promotion with PB stimulates the clonal expansion of only a minority of the single $PGST^+$ hepatocytes, as has also been demonstrated for selection in the resistant hepatocyte model.[76] Thus, at least some percentage of the single hepatocytes aberrantly expressing PGST may be the precursors for $PGST^+$ AHF that develop with administration of PB, a choline-deficient diet,[20] or AAF selection.[75] Therefore, single hepatocytes expressing PGST may represent at least one population of initiated cells in the rat liver. The nearly equivalent numbers of PGST-positive and albumin-positive hepatocytes in the analbuminemic rat liver following B[*a*]P initiation and PB promotion suggest that initiation and mutation at the PGST and albumin locus, respectively, may be similar processes. The analbuminemic rat thus may provide a unique model in which to examine initiation and mutation *in vivo* simultaneously.

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