Environmental Health Perspectives
Vol. 88, pp. 197–205, 1990

Growth-Related Alterations during Liver Carcinogenesis: Effect of Promoters

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Bromodeoxyuridine labeling of DNA, binuclearity counting, and flow cytometric analysis of isolated hepatocytes and hepatocyte nuclei has been used to assess hepatocellular growth patterns related to liver carcinogenesis. Three growth patterns can be distinguished. Mononucleating growth is observed during liver regeneration and after treatment with the tumor promoter 2-acetylaminofluorene (2-AAF) and its analogue 4-AAF. In this growth mode binucleation does not occur, resulting in a decrease in the fraction of binucleated cells. Binucleating growth is observed during normal liver development and after treatment with compounds such as phenobarbital, characterized by progressive polyploidization and maintenance of a binucleated cell fraction. Diploid growth is the growth pattern of neoplastic liver hepatocytes. Most of the cells in neoplastic lesions (foci, nodules, and carcinomas) are diploid, in contrast to the normal liver. Diploid tumor cells have a much higher proliferative activity than tetraploid tumor cells, suggesting that the latter may posses a limited growth potential that makes abrogation of binucleation proliferatively advantageous.

Introduction

Cancer is basically a growth control disease, and liver cancer is no exception. In experimental liver carcinogenesis, altered growth control has been described at several stages. Early, phenotypically altered hepatocellular foci, induced by an initiating carcinogen such as diethylnitrosamine (DEN), exhibit in general a normal or moderately elevated rate of proliferation during the first few months of their existence. The altered foci respond relatively normally to various liver growth stimuli (*1–5*). However, the focal hepatocytes proliferate particularly vigorously in response to the liver tumor promoter 2-acetylaminofluorene (2-AAF), whereas normal hepatocytes do not (*5–7*). A minority of the foci (those with the most complex phenotype) appear to be constitutively hyperproliferative and are probably precursors of the benign neoplastic nodules that eventually develop (*4*). These nodules have a high rate of proliferation and cell turnover and show an extended regenerative response (*8*).

At the hepatocellular carcinoma stage, a multitude of growth-related alterations have been described. These include expression of activated *ras* oncogenes (*9,10*); hyperexpression of c-*myc* and other proto-oncogenes (*11–13*); growth receptor alterations (*14–16*), and synthesis of growth factors not normally produced by adult hepatocytes, such as IGF-II (*17*), bFGF (*18*), bombesin (*19*), and others (*20,21*). Some of these changes may be regarded as aspects of tumor progression, contributing to increasing tumor growth rate and autonomy through the establishment of autocrine circuits.

Despite our increasing knowledge about peripheral effector alterations in hepatocellular tumors, the basic mechanisms of liver growth control remain poorly understood. Growth in a specialized tissue is an integral part of the specific differentiation program of that tissue, and in the adult liver, hepatocytic proliferation is uniquely characterized by extensive polyploidization (*22*). This pattern is replaced by nonpolyploidizing growth during the rapid regeneration following partial hepatectomy (*23*), whereas in hepatocellular tumors growth occurs mainly by divisional proliferation of diploid cells (*24*). The functional significance and selective control of these various growth patterns is not known.

Growth pattern alterations are detectable at the early stages of DEN/2-AAF-induced liver carcinogenesis (*25*). We have sought to analyze growth patterns throughout the carcinogenic development process, making comparisons with the proliferative strategies employed by normal hepatocytes. The effects of liver tumor promoters have been given particular emphasis. Although most tumor promoters are believed to act primarily as specific growth stimulants, other mechanisms of action cannot be excluded (*26,27*). In the case of 2-AAF, it has been suggested that the growth of abnormal cells might be stimulated indirectly, depending on cytotoxic inhibition of normal hepatocytes and the release of a compensatory regenerative signal (*28*). Analysis of hepatocellular growth patterns following treatment

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with 2-AAF and related compounds could help to elucidate the mechanism of action of this important liver tumor promoter.

Promotion of Primary and Secondary Liver Carcinogenesis by 2-AAF: Stimulation of Neoplastic Cell Growth

In most of our work we have used rats of the inbred Wistar Kyoto strain in order to permit transplantation experiments. Our carcinogen treatment protocol used the very effective combination of two-thirds partial hepatectomy (PH) and DEN (50 mg/kg) for initiation (*29*), followed after 1 week of regeneration by a 4-week treatment with dietary 2-AAF (0.01–0.02%) as a promoter (*25*). It should be noted that 2-AAF is a carcinogenic compound, with potentially initiating as well as promoting properties. When given at high doses for a long time 2-AAF can be strongly tumorigenic (*30*), but when administered at moderate doses (0.01–0.02% in the diet for 4 weeks or less) in the absence of additional proliferative stimuli, carcinoma formation is very late in occurrence and low in incidence (*31*).

The promoting effect of 2-AAF was detectable by the end of the treatment period as an increase in the number of enzymatically altered hepatocytes staining positively for the enzyme γ-glutamyltranspeptidase (GGT) (*31*). The GGT-positive cells, which have a focal distribution in the intact liver, can be precisely quantitated in hepatocyte suspensions prepared by collagenase perfusion (*25*). While virtually undetectable in noninitiated or nonpromoted animals, as many as 30% of the hepatocytes were GGT-positive in rats that had received the complete carcinogen treatment (Table 1).

Promotion by 2-AAF was also evident at the tumor level. Four months after initiation, the number of large neoplastic nodules (≥ 1 mm in diameter) was 10 times higher in rats given the promoter, and at 8 months the promoted animals had 3 times as many hepatocellular carcinomas as the nonpromoted ones (Table 1). Considering that the 2-AAF treatment had been terminated after 5 weeks, the effect of this promoter on liver carcinogenesis can be regarded as effectively irreversible. The irreversibility was furthermore reflected in the long-term persistence of a major fraction of the enzyme-altered (GGT-positive) hepatocytes (*31*).

If 2-AAF promoted liver carcinogenesis by stimulating the growth of neoplastic hepatocytes, we might perhaps expect 2-AAF responsiveness to be retained even in established hepatocarcinoma cells. To examine this possibility independently of any specific early-stage promotion, cell suspensions from primary hepatocellular carcinomas were transplanted by intraportal injection (*32*) to syngeneic recipients. In the host livers, outgrowth of secondary carcinomas was indeed strongly promoted by 2-AAF as well as by another liver tumor promoter, phenobarbital (Table 2).

2-AAF would thus seem to act as a stimulant of neoplastic cell growth, much like the other known liver tumor promoters (*3*). By virtue of its ability to inhibit normal hepatocyte proliferation following a partial hepatectomy (*5,6*), 2-AAF may show greater selectivity than other tumor promoters, which also effectively induce hyperplasia of the normal liver (*3*). The fact that even established cancer cells are stimulated provides a somewhat simplified concept of tumor promotion, since no stage-specific mechanisms need to be invoked. Promoters are simply growth stimulants, and any specificity in terms of extent or duration of the growth response will have to reside with the responding cell.

Table 1. Promoting effect of 2-AAF on the development of preneoplastic, GGT-positive hepatocytes, neoplastic nodules, and hepatocellular carcinomas in Wistar Kyoto rats initiated by PH and DEN (*31*).

Lesion type	Time after PH + DEN	Without 2-AAF[a]	With 2-AAF[a]
GGT-positive cells, %	6 weeks	0.0 ± 0.0 (2)	29.1 ± 3.8 (4)*
Nodules (≥ 1 mm)/animal	4 months	1.4 ± 0.5 (5)	14 ± 3 (11)†
Hepatocellular carcinomas/animal	8 months	0.8 ± 0.3 (6)	2.4 ± 0.4 (8)†

[a] Values are means ± SE; number of animals given in parentheses.
*$p < 0.001$, Student's *t*-test.
†$p < 0.02$, Student's *t*-test.

Table 2. Effect of tumor promoters on outgrowth of secondary hepatocellular carcinomas.[a]

Recipient treatment	Harvest time	% cancer incidence (animals/total)	No. of tumors/ animal	Total tumor mass/ animal, mg
None (PH)	2 months	62 (13/21)	9.5 ± 6.3	31 ± 17
2-AAF	2 months	90 (18/20)	42.0 ± 12.6*	398 ± 159†
PB	2 months	100 (19/19)‡	101.8 ± 35.1†	1608 ± 721*

[a] Donor rats received full carcinogen treatment (PH + DEN + 2-AAF), and after 8 months cell suspensions were prepared from hepatocellular carcinomas and transplanted by intraportal injection (of 500,000 viable cells) to partially hepatectomized (PH) syngeneic recipients. The recipients were subjected to secondary promotion with 2-AAF or phenobarbital (PB) as indicated. From Seglen et al. (*39*), with minor corrections.
*$p < 0.05$, Student's *t*-test.
†$p < 0.02$, Student's *t*-test.
‡$p < 0.005$, Student's *t*-test.

Direct or Indirect Growth Stimulation by 2-AAF?

The observations that 2-AAF promoted the nodular outgrowth of DEN-altered cells particularly effectively in the partially hepatectomized liver, while simultaneously suppressing the proliferation of normal hepatocytes, led Farber (*6*) to propose differential cytotoxicity as the driving force of promotion. Hepatocellular tumors—and presumably their cellular precursors—are characteristically drug resistant due to high expression of the multidrug resistance (*mdr*) gene (*33*) and various drug-metabolizing enzymes (*34*). Initiated cells might therefore resist the cytotoxic effects exerted by 2-AAF upon normal hepatocytes in the partially hepatectomized liver (*6,35*), enabling the initiated cells to proliferate selectively under the prolonged regenerative stimulus provided by the cytotoxic conditions. However, several authors found that 2-AAF could promote liver carcinogenesis even under conditions where no hepatotoxicity was evident, as when the drug was administered in the absence of or after a partial hepatectomy (*31,36*). Such results would seem to indicate that 2-AAF can promote by a mechanism independent of cytotoxicity.

2-AAF exhibits significant systemic toxicity, as indicated by a 20% lower body weight in treated animals even 3 weeks after cessation of treatment (Table 3). However, the drug was apparently not detectably hepatotoxic in our experimental protocol, since the liver grew at the same rate as in control animals throughout the treatment period (*31*). The liver weight, protein content, and DNA content was thus normal 3 weeks after cessation of treatment (Table 3). The lack of 2-AAF hepatotoxicity contrasts with the previously observed suppression of normal hepatocyte growth in partially hepatectomized Fischer rats (*6*), and may reflect a lower 2-AAF sensitivity in the Wistar Kyoto strain as well as the fact that treatment in the present experiments was begun some time after partial hepatectomy. Hepatocytes have been found to exhibit increased drug resistance after liver regeneration (*37*), possibly because of elevated expression of the *mdr* gene (*38*). In any case, promotion of carcinogenesis by 2-AAF in our experiments would seem to be effected by a noncytotoxic mechanism.

2-AAF Alters the Hepatocellular Growth Pattern

Although 2-AAF neither inhibits nor stimulates proliferation of normal hepatocytes under our experimental conditions, it can nevertheless be shown to affect hepatocytic growth (*23*). A combined flow cytometric and light microscopic analysis of hepatocytic cell suspensions and isolated nuclei (Table 4) revealed that 2-AAF treatment markedly altered hepatocellular ploidy distributions (*23*). The most striking changes were a decrease in the fractions of binucleated cells and an increase in the diploid cell fraction. These changes resemble the ones observed during liver regeneration (Table 4) and can best be described as a switch from a binucleating (polyploidizing) to a mononucleating (nonpolyploidizing) mode of growth (*23*). Another liver tumor promoter, phenobarbital, has been found to stimulate net growth (limited hyperplasia) of the normal liver without altering hepatocellular ploidy distributions (*39*). Different tumor promoters would therefore appear capable of eliciting different proliferation programs in the liver.

Table 3. Systemic toxicity, but no hepatotoxicity, of 2-AAF given to young male rats 1 to 5 weeks after partial hepatectomy.[a]

Parameter	Without 2-AAF	With 2-AAF
Body weight, g	320.0 ± 10.4	264.8 ± 4.9*
Liver weight, g	9.7 ± 0.4	9.7 ± 0.4
Liver dry mass, mg	2439 ± 159	2362 ± 81
Liver protein, mg	1842 ± 102	1689 ± 55
Liver DNA, mg	15.8 ± 0.7	15.3 ± 0.2

[a] Rats weighing 70 g were subjected to two-thirds partial hepatectomy, given 0.02% dietary 2-AAF from 1 to 5 weeks, and sacrificed at 8 weeks. Each value is the mean ± SE of three animals.
* $p < 0.01$, Student's *t*-test.

Table 4. Similar alterations in hepatocellular ploidy distributions after 2-AAF treatment and liver regeneration.[a]

Group	2*N*	2 × 2*N*	4*N*	2 × 4*N*	8*N*
Control	11.2	10.9	54.6	13.2	10.0
2-AAF	38.5	2.7	53.5	1.0	4.4
Before regeneration	25.7	22.6	39.8	8.7	3.1
After regeneration	38.1	4.9	51.5	1.7	3.8

[a] Cellular and nuclear ploidy measurements were made immediately before or 1 week after partial hepatectomy, or at 6 weeks with or without intermediary 2-AAF feeding (1–5 weeks). Calculated cell class distributions from Saeter et al. (*23*).

Stimulation of Hepatocellular Growth by the Analogue 4-AAF

The ability of 2-AAF to alter the liver growth pattern without actually stimulating liver growth could possibly be the net result of two opposing effects: a specific stimulation of mononucleating growth and a simultaneous inhibition of binucleating growth (or a more general suppression of hepatocytic proliferation) masking the stimulation. In an attempt to examine this hypothesis indirectly, we administered the analogue 4-AAF, which has been reported to be noncytotoxic (*40*) and to stimulate DNA synthesis in normal liver (*41*). As shown in Table 5, 4-AAF, like phenobarbital (but unlike 2-AAF), stimulated liver growth as measured by either mass, protein content, or DNA content.

To see if 4-AAF could alter the hepatocellular growth pattern in a manner similar to 2-AAF, we gave rats a single high dose of 4-AAF and measured the fraction of binucleated hepatocytes, the most sensitive indicator of changes of this kind, at different time points during the following 2 days. Hepatocytic proliferation activity (DNA labeling index) was simultaneously monitored immunocytochemically (*42*) as the incorporation of bromodeoxyuridine (BrdU) into hepatocyte nuclei. BrdU

Table 5. Liver hyperplasia induced by 2-AAF, 4-AAF, and phenobarbital.[a]

Treatment	Liver weight, g	Protein, g/liver	DNA, mg/liver
Control (2 weeks)	6.00 ± 0.22 (4)	1.24 ± 0.02 (4)	15.2 ± 0.3 (4)
2-AAF (0.01%, 2 weeks)	5.61 ± 0.32 (4)	1.16 ± 0.04 (4)	14.9 ± 0.6 (4)
4-AAF (0.1%, 2 weeks)	6.90 ± 0.26 (4)*	1.38 ± 0.04 (4)*	19.6 ± 0.4 (4)†
Phenobarbital (0.04%, 2 weeks)	7.66 ± 0.19 (4)‡	1.50 ± 0.05 (4)†	20.3 ± 0.2 (4)†

[a] Rats 4 weeks old, weighing 70 g, were given dietary 2-AAF, 4-AAF, or phenobarbital for 2 weeks. Values are the means ± SE; number of animals given in parentheses.
*$p < 0.05$, Student's t-test.
†$p < 0.001$, Student's t-test.
‡$p < 0.005$, Student's t-test.

was administered in the form of solid tablets (*43*) deposited SC at regular intervals, using labeling periods from 8 to 60 hr.

About 30 hr after oral administration of 4-AAF, a precipitous drop in the binucleated fraction was observed (Table 6). Division of binucleated cells gives rise to mononucleated progeny of higher ploidy (*22*); these cells therefore need to be formed by *de novo* binucleation at the same rate as they divide to remain as a constant fraction of the hepatocyte population. The stimulation of proliferation by 4-AAF clearly upsets this balance. There are two basically different mechanisms by which binucleated cells might disappear: by selective stimulation of their proliferation in excess of replenishment through binucleation, or by an absolute or relative inhibition of binucleation. In the latter case, the fraction of binucleated cells would decrease both by dilution with mononucleated cells and as a result of their own division. The results in Table 6 tend to support the second alternative: the binucleated cells are not specifically stimulated by 4-AAF; their labeling index is in fact significantly lower than that of the mononucleated cells. 4-AAF would thus seem to induce a mononucleating type of growth, causing binucleated to disappear by a combination of dilution and division.

Long-term treatment with 4-AAF caused an even more pronounced reduction in the fraction of binucleated hepatocytes both in normal and DEN-treated liver (Table 7). These alterations were similar to those observed with 2-AAF, whereas phenobarbital (not shown) had no effect under the same conditions.

Table 6. Reduced fraction of binucleated hepatocytes after 4-AAF treatment: proliferative activity of binucleated and mononucleated cells.[a]

Time after 4-AAF	No. of animals	Percentage of binucleated hepatocytes	Labeling index, %	
			Mononucleated	Binucleated
7–15 hr	3	27.1 ± 1.7	0.6 ± 0.6	0.5 ± 0.5
15–31 hr	6	27.1 ± 1.4	17.7 ± 2.9	7.2 ± 1.6*
31–47 hr	6	14.9 ± 1.6	25.2 ± 1.3	7.5 ± 2.4†

[a] Rats were given a single dose of 4-AAF (1000 mg/kg), and a pulse of BrdU 8 hr before sacrifice. Binuclearity and labeling index (percentage of BrdU-positive cells) were measured by light microscopy. The two latter time intervals include pooled values from pulse periods each (15–23 hr and 23–31 hr in the second interval; 31–39 hr and 39–47 hr in the third). Each value is the mean ± SE of three or six animals.
* Binucleated labeling index significantly lower than mononucleated labeling index at $p < 0.01$, Student's t-test.
† Binucleated labeling index significantly lower than mononucleated labeling index at $p < 0.001$, Student's t-test.

Table 7. Effect of 4-week treatment with 2-AAF or 4-AAF on hepatocytic binuclearity, labeling index, and percentage of diploid nuclei.[a]

Treatment	No. of animals	% Binucleated hepatocytes	% Diploid nuclei	Hepatocytic labeling index
None	3	20.1 ± 0.6	44.2 ± 1.6	0.6 ± 0.3
2-AAF	3	6.7 ± 0.4	45.9 ± 1.5	3.1 ± 0.9
4-AAF	3	3.9 ± 0.1	46.2 ± 1.1	2.4 ± 0.6
DEN	4	18.4 ± 1.2	51.5 ± 1.4	1.1 ± 0.3
DEN + 2-AAF	4	4.3 ± 1.0*	64.4 ± 4.1†	11.7 ± 1.3*
DEN + 4-AAF	4	4.8 ± 0.7*	68.0 ± 1.0*	9.2 ± 2.8†

[a] Partially hepatectomized rats were given a single initiating dose of DEN and/or 2-AAF or 4-AAF for 4 weeks as indicated. Binucleated hepatocytes were counted in the light microscope; nuclear ploidy distributions by flow cytometry, and labeling index as the percentage of BrdU-positive hepatocytes (microscope counting of isolated cells) after a 60-hr incorporation *in vivo*, followed by a 12-hr chase. Each value is the mean ± SE of three or four animals.
*$p < 0.001$ versus DEN alone, Student's t-test.
†$p < 0.05$ versus DEN alone, Student's t-test.

The growth response to 4-AAF was a limited hyperplasia, similar to what has been observed with other liver growth stimulants (*44*). After 4 weeks of treatment, the hepatocytic labeling index was thus only insignificantly elevated in noninitiated livers (Table 7). In DEN-initiated livers, on the other hand, the labeling index was markedly increased both in 4-AAF-treated and 2-AAF-treated livers. An elevated fraction of diploid hepatocyte nuclei was also evident with both agents (Table 7). In the case of 2-AAF, these changes are known to reflect the proliferation of focal hepatocytes (*5,6*), most of which are diploid (*23,45–47*), but it would probably be premature to conclude on the basis of these preliminary data that 4-AAF similarly promotes the outgrowth of altered hepatocytes. The eventual classification of 4-AAF as a tumor promoter will have to await a demonstration of its actual stimulation of the formation of hepatocellular tumors. Nevertheless, it seems clear that 4-AAF in its role as a liver growth stimulant induces a mononucleating growth pattern resembling that observed after 2-AAF treatment. A similar pattern change has been observed after treatment with a liver growth-stimulating and tumor-promoting

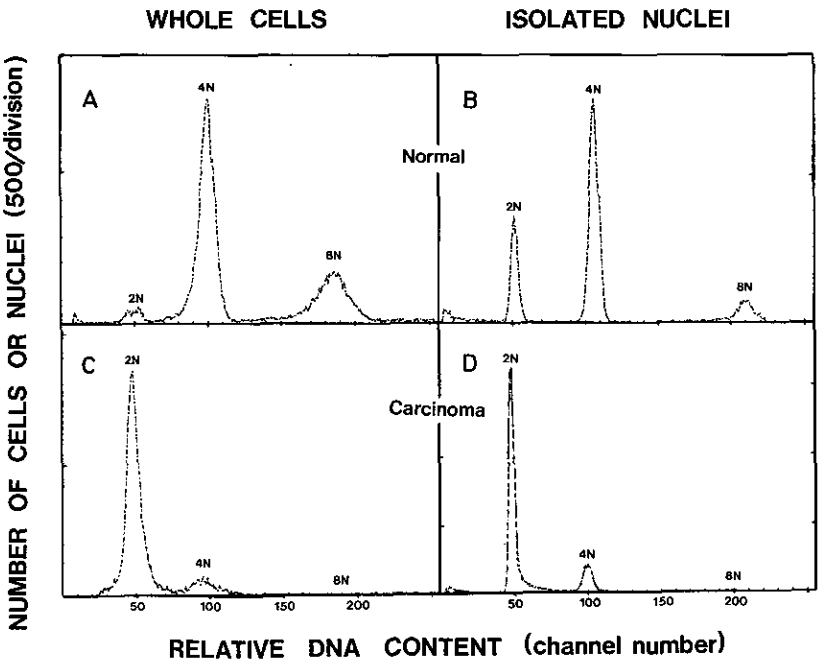

FIGURE 1. Flow cytometric DNA distributions of cells and nuclei from control livers 6 weeks after partial hepatectomy (PH) (*A,B*) and from a hepatocellular carcinoma 32 weeks after initiation of PH/DEN/2-AAF treatment (*C,D*). Modified from Sæter et al. (*24*).

steroid, cyproterone acetate (*48*). These results strengthen the notion that even 2-AAF may have intrinsic growth-stimulatory properties that are independent of (but in the normal liver masked by) its cytotoxic actions. Whether 2-AAF and 4-AAF actually share a common mechanism of growth stimulation remains to be shown. It should be noted that 4-AAF is needed at 10 times higher concentrations than 2-AAF to be maximally effective, and the two compounds differ both in their receptor affinities (*49*) and in their relative abilities to induce various drug-metabolizing enzymes (*50*). It is thus conceivable that an AAF receptor with higher affinity for 2-AAF than for 4-AAF may mediate the stimulation of neoplastic cell growth.

Diploid Growth Pattern of Hepatocellular Tumors

Hepatocellular tumors are basically hyperproliferative lesions, but in addition to the elevated growth rate, their abnormal growth is reflected in the pattern of cellular growth. Whereas the normal liver grows predominantly by progressive polyploidization, both neoplastic nodules and hepatocellular carcinomas have a strongly reduced tendency to polypoidize and instead grow mainly by divisional proliferation of diploid cells (*24,25*). This neoplastic growth patterns is readily observed by flow cytometric analysis of isolated tumor cells and nuclei (Fig. 1). In normal liver tissue, only about 10% of the hepatocytes (and 20–40% of the hepatocyte nuclei, depending on the strain- and age-dependent size of the binuclear fraction) are diploid, while nodules on average contain 70% diploid cells and carcinomas 80% diploid cells (*24*). We have analyzed hepatocellular carcinomas induced by a variety of carcinogenic regimens, and found a predominantly diploid growth pattern in all cases (*39*). Even in human liver cancer, a reduced polyploidization tendency is evident, although the moderate degree of polyploidy in the normal human liver tissue makes the difference somewhat less striking than in the rat (*51*). The emergence of increasing numbers of diploid cells is observable at an early stage of carcinogenesis (*23,25*) (Table 6).

The function of liver polyploidy and its abrogation in liver tumors is not entirely clear. A significant correlation between tumor size and fractional content of diploid cells in synchronous liver nodules suggests that polyploidization is associated with a reduced growth potential (*24*). Polyploidization is an irreversible, progressively terminal differentiation process (*22*); the polyploid cells are accordingly incapable of generating diploid progeny and can presumably only undergo a limited number of divisions (*52*). The switch to a diploid, divisional growth pattern in tumors would maintain a larger population of potential stem cells and hence a higher capacity for continual, unlimited growth. It is noteworthy that although abnormal preneoplastic foci of tetraploid hepatocytes have been observed (*45*), tetraploid liver tumors have never been reported, supporting the contention of a limited proliferation potential in polyploid cells.

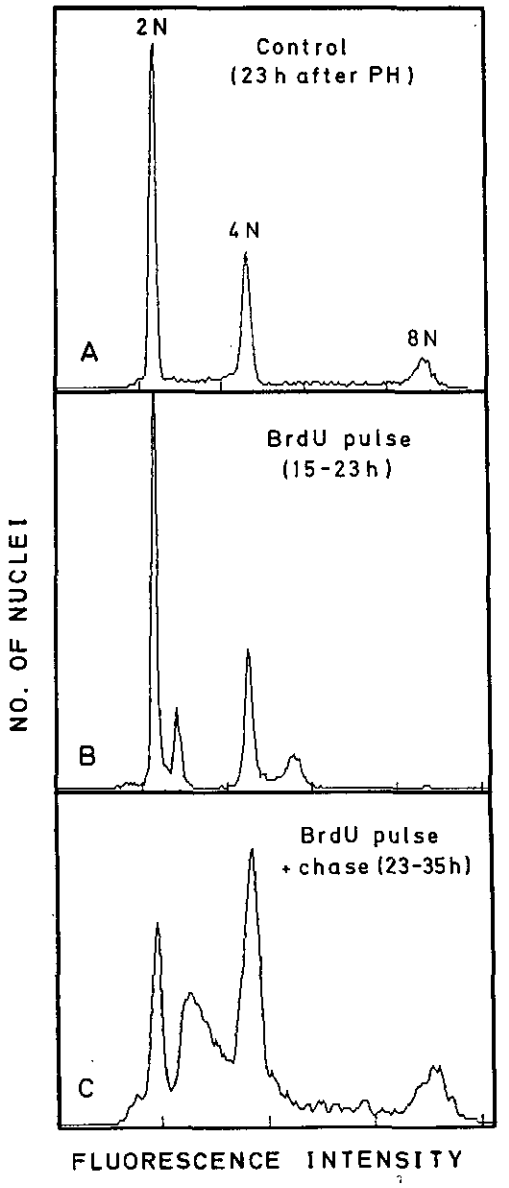

FIGURE 2. Flow cytometric DNA distributions of hepatocytic nuclei from regenerating rat liver stained with the fluorescent dye Hoechst 33258. (*A*) Unquenched pattern, 23 hr after partial hepatectomy (PH). (*B*) Animals treated with BrdU for 8 hr before sacrifice, 23 hr after PH. Notice strongly quenched 4*N* and 8*N* subpopulations (BrdU-labeled nuclei in late S and G_2 phase) located far below the respective unquenched peaks. (*C*) 8 hr-BrdU pulse followed by 12 hr chase; sacrifice 35 hr after PH. Notice quenched 2*N* and 4*N* subpopulations (divided BrdU-labeled nuclei) positioned below the respective unquenched peaks; also notice the reappearance of the unquenched 8*N* peak.

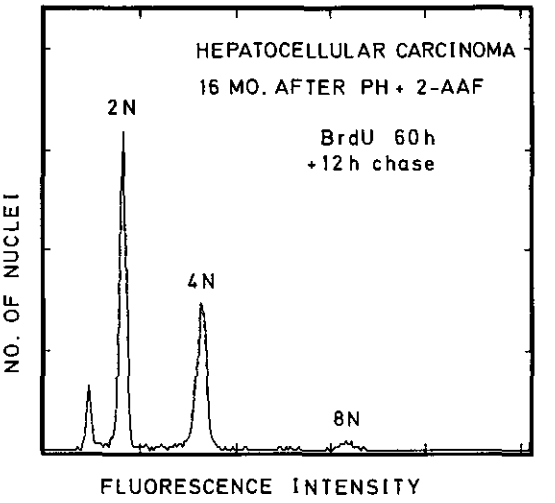

FIGURE 3. Flow cytometric DNA distributions of nuclei from hepatocellular carcinoma 16 months after PH and 2-AAF treatment. Hoechst 33258 staining; 60 hr BrdU incorporation and 12 hr chase. Notice quenched peak (divided BrdU-labeled diploid nuclei) below the unquenched 2*N* peak.

Table 8. Hepatocellular growth patterns.

Mononucleating growth (regeneration, 2-AAF, 4-AAF, steroids)
Binucleating growth (development, phenobarbital, chlorinated organochemicals)
Diploid growth (neoplastic growth)

Proliferative Activity of Different Ploidy Classes in Normal Liver and Hepatocellular Carcinoma

To further investigate the proliferative activity of the different hepatocytic ploidy classes, we used flow cytometric analysis of BrdU-labeled cells. The principle of the method is that DNA-incorporated BrdU will reduce the binding and hence quench the fluorescence yield from the DNA-staining dye Hoechst 33258, giving rise to intermediary peaks of DNA-synthesizing cells (S phase and G_2) in flow cytometric DNA histograms (*53*).

Figure 2 shows how this methodology has been applied to normal, regenerating livers. Twenty-three hours after PH, many hepatocytes are actively synthesizing DNA as indicated by the elevated baseline fluorescence between the major nuclear DNA peaks (Fig. 2*A*). In histograms from BrdU-labeled cells, the DNA-synthesizing nuclei are evident as one quenched tetraploid peak (in the hyperdiploid position) and one quenched octoploid peak (in the hypertetraploid position), representing labeled nuclei of diploid and tetraploid origin, respectively, in late S and G_2 phase of the cell cycle (Fig. 2*B*). It is noteworthy that the octoploid peak observed in Figure 2*A* is virtually absent in BrdU-labeled nuclei, showing that it represents a tetraploid G_2 fraction rather than a true octoploid (i.e., G_1) population. There is no fluorescence in the hyperoctoploid region, i.e., proliferating 8N nuclei are not detectably present.

The fate of the labeled nuclei after cell division can be followed by allowing a chase period after cessation of BrdU incorporation. Figure 2*C* shows that most of the labeled tetraploid nuclei have been shifted to a subtetraploid position (i.e., quenched tetraploid DNA content), indicating that nuclear division has occurred. The

appearance of quenched diploid nuclei is less extensive, reflecting the fact that a major fraction of the diploid nuclei originally reside in binucleated cells and give rise to tetraploid nuclei, which retain their position in the histogram following DNA synthesis and division. The quenched tetraploid nuclei observed after a chase thus have mixed origins that cannot be resolved with the present method. The reappearance of the unquenched $8N$ peak during the chase period reflects the entry of tetraploid nuclei into S and G_2 in the absence of BrdU incorporation.

When BrdU/Hoechst flow cytometry is applied to hepatocellular tumors, an entirely different result is obtained. We have deliberately chosen to analyze slow-growing tumors from old rats, in which a significant fraction of tetraploid nuclei are present. As shown in Figure 3, following a 60-hr BrdU pulse and a 12-hr chase, labeled nuclei are found virtually exclusively in the quenched diploid region of the histogram. About one-half of these are nonparenchymal nuclei (which constitute a significant fraction of the labeled tumor nuclei after long-term labeling), but the remaining half are of bona fide hepatocellular (tumor) origin. In other words, only the diploid tumor nuclei show significant proliferative activity. This phenomenon, which has been consistently observed in the tumors we have analyzed, supports the notion of tetraploid cells as a subterminally differentiated phenotype with a limited growth potential. In hepatocellular tumors, this growth potential may eventually become exhausted, leaving the tetraploid cells as a proliferatively silent (but long-lived) subpopulation. Alternatively, some renewal of the tetraploid population may take place, but at a rate too low to be detected by the present methods.

In contrast, the diploid hepatocellular tumor nuclei have a labeling index that is high compared to the rate of tumor growth. The extensive cellular turnover that has been described in hepatocellular tumors (*8*) would thus seem to be confined to this diploid fraction. The proliferative polymorphism of hepatocellular tumors may be an aspect of tumor differentiation worthy of further study. In any event, the limitation of detectable proliferative activity to the diploid fraction of the tumor nuclei underscores the importance of a diploid growth pattern as a probably essential aspect of the neoplastic liver cell phenotype.

Our studies thus suggest that at least three different modes of hepatocellular proliferation may exist (Table 8). Mononucleating growth is observed during liver regeneration and after treatment with 2-AAF or 4-AAF and probably after steroid treatment (*48*). This type of growth is characterized by divisional proliferation of both diploid and tetraploid hepatocytes, but there is essentially no binucleation and hence no progressive polyploidization of the tissue (apart from a possible transient elevation of the tetraploid fraction as residual binucleated cells undergo division). Binucleating growth is occurring during normal liver development and after stimulation by compounds such as phenobarbital (*39*) or chlorinated organochemicals (*48,54*). Binucleation is a regular feature of this growth pattern, resulting in progressive tissue polyploidization and the maintenance of a binucleated cell fraction. Diploid growth, i.e., divisional proliferation of diploid hepatocytes, is the pattern characteristic of neoplastic growth. It may have the virtue of maintaining a high growth capacity as well as a large population of potential stem cells capable of expressing the further mutational changes thought to be involved in tumor progression.

The possibility should be considered that the mononucleating growth pattern may gradually develop into a predominantly diploid growth pattern as a result of exhaustion of the proliferative potential of the tetraploid cells. If so, the number of basic hepatocellular growth patterns may be reduced by one. In the adult rat liver, binucleating growth would appear to be the constitutive pattern, while the other modes of growth are mobilized by specific stimuli. Replacement of binucleating by mononucleating growth as the new constitutive pattern may be an early and essential event in liver carcinogenesis.

This work has been generously supported by the Norwegian Cancer Society and the Norwegian Council for Science and the Humanities. The skillful technical assistance of Åse Bakketun and Roger Aarvik is gratefully acknowledged. We also thank Trond Stokke for his invaluable help with the flow cytometric measurements.

REFERENCES

1. Rabes, H. M., Scholze, P., and Jantsch, B. Growth kinetics of diethylnitrosamine-induced, enzyme-deficient "preneoplastic" liver cell populations *in vivo* and *in vitro*. Cancer Res. 32: 2577–2586 (1972).
2. Rabes, H. M., and Szymkowiak, R. Cell kinetics of hepatocytes during the preneoplastic period of diethylnitrosamine-induced liver carcinogenesis. Cancer Res. 39: 1298–1304 (1979).
3. Schulte-Hermann, R., Ohde, G., Schuppler, J., and Timmermann-Trosiener, I. Enhanced proliferation of putative preneoplastic cells in rat liver following treatment with the tumor promoters phenobarbital, hexachlorocyclohexane, steroid compounds and nafenopin. Cancer Res. 41: 2556–2562 (1981).
4. Peraino, C., Staffeldt, E. F., Carnes, B. A., Ludeman, V. A., Blomquist, J. A., and Vesselinovitch, S. D. Characterization of histochemically detectable altered hepatocyte foci and their relationship to hepatic tumorigenesis in rats treated once with diethylnitrosamine or benzo(*a*)pyrene within one day after birth. Cancer Res. 44: 3340–3347 (1984).
5. Tatematsu, M., Aoki, T., Kagawa, M., Mera, Y., and Ito, N. Reciprocal relationship between development of glutathione S-transferase positive liver foci and proliferation of surrounding hepatocytes in rats. Carcinogenesis 9: 221–225 (1988).
6. Solt, D. B., Medline, A., and Farber, E. Rapid emergence of carcinogen-induced hyperplastic lesions in a new model for the sequential analysis of liver carcinogenesis. Am. J. Pathol. 88: 595–618 (1977).
7. Kitagawa, T. Sequential phenotypic changes in the hyperplastic areas during hepatocarcinogenesis in the rat. Cancer Res. 36: 2534–2539 (1976).
8. Rotstein, J., Sarma, D. S. R., and Farber, F. Sequential alterations in growth control and cell dynamics of rat hepatocytes in early precancerous steps in hepatocarcinogenesis. Cancer Res. 46: 2377–2385 (1986).
9. Reynolds, S. H., Stowers, S. J., Patterson, R. M., Maronpot, R. R., Aaronson, S. A., and Anderson, M. W. Activated oncogenes in B6C3F1 mouse liver tumors: implications for risk assessment. Science 237: 1309–1316 (1987).
10. McMahon, G., Hanson, L., Lee, J.-J., and Wogan, G. N. Identification of an activated c-Ki-*ras* oncogene in rat liver tumors

induced by aflatoxin B_1. Proc. Natl. Acad. Sci. USA 83: 9418–9422 (1986).
11. Tashiro, F., Morimura, S., Hayashi, K., Makino, R., Kawamura, H., Horikoshi, N., Nemoto, K., Ohtsubo, K., Sugimura, T., and Ueno, Y. Expression of the c-Ha-*ras* and c-*myc* genes in aflatoxin B_1-induced hepatocellular carcinomas. Biochem. Biophys. Res. Commun. 138: 858–864 (1986).
12. Corral, M., Paris, B., Guguen-Guillouzo, C., Corcos, D., Kruh, J., and Defer, N. Increased expression of the N-*myc* gene during normal and neoplastic rat liver growth. Exp. Cell Res. 174: 107–115 (1988).
13. Corral, M., Tichonicky, L., Guguen-Guillouzo, C., Corcos, D., Raymondjean, M., Paris, B., Kruh, J., and Defer, N. Expression of c-*fos* oncogene during hepatocarcinogenesis, liver regeneration and in synchronized HTC cells. Exp. Cell Res. 160: 427–434 (1985).
14. Lev-Ran, A., Carr, B. I., Hwang, D. L., and Roitman, A. Binding of epidermal growth factor and insulin and the autophosphorylation of their receptors in experimental primary hepatocellular carcinomas. Cancer Res. 46: 4656–4659 (1986).
15. Brønstad, G. O., Christoffersen, T., Johansen, E. J., and Øye, I. Effect of prostaglandins and hormones on cyclic AMP formation in rat hepatomas and liver tissue. Br. J. Cancer 38: 737–743 (1978).
16. Benbrook, D., Lernhardt, E., and Pfahl, M. A new retinoic acid receptor identified from a hepatocellular carcinoma. Nature 333: 669–672 (1988).
17. Norstedt, G., Levinovitz, A., Möller, C., Eriksson, L. C., and Andersson, G. Expression of insulin-like growth factor I(IGF-I) and IGF-II mRNA during hepatic development, proliferation and carcinogenesis in the rat. Carcinogenesis 9: 209–213 (1988).
18. Presta, M., Moscatelli, D., Joseph-Silverstein, J., and Rifkin, D. B. Purification from a human hepatoma cell line of a basic fibroblast growth factor-like molecule that stimulates capillary endothelial cell plasminogen activator production, DNA synthesis, and migration. Mol. Cell. Biol. 6: 4060–4066 (1986).
19. Seglen, P. O., Skomedal, H., Sæter, G., Schwarze, P. E., and Nesland, J. M. Neuroendocrine dysdifferentiation and bombesin production in carcinogen-induced hepatocellular rat tumors. Carcinogenesis 10: 21–25 (1989).
20. Luetteke, N. C., and Michalopoulos, G. K. Partial purification and characterization of a hepatocyte growth factor produced by rat hepatocellular carcinoma cells. Cancer Res. 45: 6331–6337 (1985).
21. Ove, P., Coetzee, M. L., Scalamogna, P., Francavilla, A., and Starzl, T. E. Isolation of an autocrine growth factor from hepatoma HTC-SR cells. J. Cell. Physiol. 131: 165–174 (1987).
22. Brodsky, W. Y., and Uryvaeva, I. V. Cell polyploidy: its relation to tissue growth and function. Int. Rev. Cytol. 50: 275–332 (1977).
23. Sæter, G., Schwarze, P. E., and Seglen, P. O. Shift from polyploidizing to non-polyploidizing growth in carcinogen-treated rat liver. J. Natl. Cancer Inst. 80: 950–958 (1988).
24. Sæter, G., Schwarze, P. E., Nesland, J. M., Juul, N., Pettersen, E. O., and Seglen, P. O. The polyploidizing growth pattern of normal rat liver is replaced by divisional, diploid growth in hepatocellular nodules and carcinomas. Carcinogenesis 9: 939–945 (1988).
25. Schwarze, P. E., Pettersen, E. O., Shoaib, M. C., and Seglen, P. O. Emergence of a population of small, diploid hepatocytes during hepatocarcinogenesis. Carcinogenesis 5: 1267–1275 (1984).
26. Schulte-Hermann, R. Tumor promotion in the liver. Arch. Toxicol. 57: 147–158 (1985).
27. Moore, M. A., and Kitagawa, T. Hepatocarcinogenesis in the rat: the effect of promoters and carcinogens *in vivo* and *in vitro*. Int. Rev. Cytol. 101: 125–173 (1986).
28. Farber, E. Cellular biochemistry of the stepwise development of cancer with chemicals: G. H. A. Clowes Memorial Lecture. Cancer Res. 44: 5463–5474 (1984).
29. Scherer, E., and Emmelot, P. Kinetics of induction and growth of precancerous liver-cell foci, and liver tumour formation by diethylnitrosamine in the rat. Eur. J. Cancer 11: 689–696 (1975).
30. Teebor, G. W., and Becker, F. F. Regression and persistence of hyperplastic hepatic nodules induced by N-2-fluorenylacetamide and their relationship to hepatocarcinogenesis. Cancer Res. 31: 1–3 (1971).
31. Sæter, G., Schwarze, P. E., Nesland, J. M., and Seglen, P. O. 2-Acetylaminofluorene promotion of liver carcinogenesis by a non-cytotoxic mechanism. Carcinogenesis 9: 581–587 (1988).
32. Sæter, G., Schwarze, P. E., Nesland, J. M., and Seglen, P. O. Diploid nature of hepatocellular tumours developing from transplanted preneoplastic liver cells. Br. J. Cancer 59: 198–205 (1989).
33. Fairchild, C. R., Ivy, S. P., Rushmore, T., Lee, G., Koo, P., Goldsmith, M. E., Myers, C. E., Farber, E., and Cowan, K. H. Carcinogen-induced *mdr* overexpression is associated with xenobiotic resistance in rat preneoplastic liver nodules and hepatocellular carcinomas. Proc. Natl. Acad. Sci. USA 84: 7701–7705 (1987).
34. Eriksson, L., Blanck, A., Bock, W., and Mannervik, B. Metabolism of xenobiotics in hepatocyte nodules. Toxicol. Pathol. 15: 27–42 (1987).
35. Jackson, C. D., and Irving, C. C. Effects of 2-acetylaminofluorene on liver cell proliferation after partial hepatectomy of female rats. Cancer Res. 33: 397–401 (1973).
36. Tatematsu, M., Shirai, T., Tsuda, H., Miyata, Y., Shinohara, Y., and Ito, N. Rapid production of hyperplastic liver nodules in rats treated with carcinogenic chemicals: a new approach for an *in vivo* short-term screening test for hepatocarcinogens. Jpn. J. Cancer Res. (Gann) 68: 499–507 (1977).
37. Carr, B. I., and Laishes, B. A. Carcinogen-induced drug resistance in rat hepatocytes. Cancer Res. 41: 1715–1719 (1981).
38. Thorgeirsson, S. S., Huber, B. E., Sorrell, S., Fojo, A., Pastan, I., and Gottesman, M. Expression of the multidrug-resistant gene in hepatocarcinogenesis and regenerating rat liver. Science 236: 1120–1122 (1987).
39. Seglen, P. O., Schwarze, P. E., and Sæter, G. Nuclear alterations in liver carcinogenesis: the role of non-polyploidizing growth. In: Chemical Carcinogenesis (F. Feo, P. Pani, A. Columbano, and R. Garcea, Eds.), Plenum Publishing Corp., New York, 1988, pp. 435–445.
40. Flaks, B. The effect of prolonged dietary administration of the non-carcinogen, 4-acetylaminofluorene on the liver of the rat. An electron microscope study. Chem.-Biol. Interact. 7: 151–164 (1973).
41. Ashby, J., Lefevre, P. A., Burlinson, B., and Beije, B. Potent mitogenic activity of 4-acetylaminofluorene to the rat liver. Mutat. Res. 172: 271–279 (1986).
42. Gonchoroff, N. J., Katzmann, J. A., Currie, R. M., Evans, E. L., Houck, D. W., Kline, B. C., Greipp, P. R., and Loken, M. R. S-phase detection with an antibody to bromodeoxyuridine. Role of DNase pretreatment. J. Immunol. Methods 93: 91–101 (1986).
43. Allen, J. W., Shuler, C. F., and Latt, S. A. Bromodeoxyuridine tablet methodology for in vivo studies of DNA synthesis. Somat. Cell Genet. 4: 393–405 (1978).
44. Schulte-Hermann, R., Timmermann-Trosiener, I., and Schuppler, J. Facilitated expression of adaptive responses to phenobarbital in putative pre-stages of liver cancer. Carcinogenesis 7: 1651–1655 (1986).
45. Sarafoff, M., Rabes, H. M., and Dörmer, P. Correlations between ploidy and initiation probability determined by DNA cytophotometry in individual altered hepatic foci. Carcinogenesis 7: 1191–1196 (1986).
46. Schwarze, P. E., Pettersen, E. O., Tolleshaug, H., and Seglen, P. O. Isolation of carcinogen-induced diploid rat hepatocytes by centrifugal elutriation. Cancer Res. 46: 4732–4737 (1986).
47. Danielsen, H. E., Steen, H. B., Lindmo, T., and Reith, A. Ploidy distribution in experimental liver carcinogenesis in mice. Carcinogenesis 9: 59–63 (1988).
48. Schulte-Hermann, R., Hoffmann, V., and Landgraf, H. Adaptive responses of rat liver to the gestagen and anti-androgen cyproterone acetate and other inducers. III. Cytological changes. Chem.-Biol. Interact. 31: 301–311 (1980).
49. Neumann, H. -G. Hepatocarcinogens. In: Experimental Hepatocarcinogenesis (M. B. Roberfroid and V. Préat, Eds.), Plenum Press, New York, 1988, pp. 5–13.
50. Åström, A., Birberg, W., Pilotti, Å., and DePierre, J. W. In-

duction of different isozymes of cytochrome P-450 and of microsomal epoxide hydrolase in rat liver by 2-acetylaminofluorene and structurally related compounds. Eur. J. Biochem. 154: 125–134 (1986).

51. Sæter, G., Lee, C. -Z., Schwarze, P. E., Ous, S., Chen, D. -S., Sung, J. -L., and Seglen, P. O. Changes in ploidy distributions in human liver carcinogenesis. J.Natl. Cancer Inst. 80: 1480–1485 (1988).
52. Lajtha, L. G. Stem cell concepts. Differentiation 14: 23–34 (1979).
53. Rabinovitch, P. S., Kubbies, M., Chen, Y. C., Schindler, D., and Hoehn, H. BrdU-Hoechst flow cytometry: a unique tool for quantitative cell cycle analysis. Exp. Cell Res. 174: 309–318 (1988).
54. van Ravenzwaay, B., Tennekes, H., Stöhr, M., and Kunz, W. The kinetics of nuclear polyploidization and tumour formation in livers of CF-1 mice exposed to dieldrin. Carcinogenesis 8: 265–269 (1987).