an extensive signal transduction network, connecting DNA damage with the activation of transcription factors and controlling the expression of genes involved in xenobiotic metabolism, DNA repair, cell cycle arrest and apoptosis. The logical consequence, therefore, is that various combinations of these responses may form the basis for carcinogenesis. Many studies have shown benefits, not harm, from low-level exposure to toxicants, a phenomenon being known as hormesis. For some chemicals tested, carcinogens are found to be similar to other toxicants in improving the outcome at low doses, although the mechanisms of their action remain unclear. Therefore, it appears very important to answer the question how carcinogens act at very low doses.

Phenobarbital (PB) is a sedative and unticolvulsant, used widely in clinical therapy for long-term treatment. It is also a well-known non-genotoxic carcinogen and tumor promoter in rodents. To elucidate a practical threshold level for the hepatopromoting effects of PB, the dose dependence was investigated using a rat liver medium-term bioassay (Ito test) (Kitano et al., 1998). PB at doses of 0, 1, 2, 4, 7.5, 15, 30, 60, 125, 250 or 500 ppm were fed to the rats. The numbers and size of GST-P-positive foci in the liver were increased dose dependently in rats given 60–500 ppm PB. However, those for doses in the range 1–7.5 ppm demonstrated a decrease as compared to the control group (0 ppm), with significant differences observed for 1 and 2 ppm. Thus, results indicated that PB exerted hormesis in the rat hepatocarcinogenicity, indicating the existence of threshold in its carcinogenicity.

In a second experiment, for clarification of the hormetic effect of PB at low doses, male 6-week-old F344 rats were treated with PB at doses of 0, 2, 15 and 500 ppm in diet for 10 or 33 weeks without 2/3 partial hepalectomy after the initiation of hepatocarcinogenesis with DEN (Kinoshita et al., 2003). The formation of GST-P-positive foci and liver tumors was inhibited at 2 ppm after 10 and 33 weeks of PB administration, respectively (Fig. 2). At week 10, generation of 8-hydroxydeoxyguanosine (8-OHdG), cellular proliferation within the areas of GST-P-positive foci, and programmed cell death, apoptosis, in background liver parenchyma were suppressed. Decrease in 8-OHdG formation by PB at low dose might be due to the elevated expression of gene for the 8-OHdG repair enzyme, oxoguanine glycosylase 1 (Ogg1). Furthermore, as detected by cDNA microarray analysis, PB at low dose enhanced mRNA expression for glutamic acid decarboxylase (GAD65), an enzyme involved in the synthesis of gamma-aminobutyric acid (GABA), and suppressed the expression of MAP kinase p38 and other intracellular kinases. In contrast, application of the chemical at a high dose enhanced GST-P-positive foci development, tumor multiplicity, generation of hydroxyl radicals, 8-OHdG levels, CYP2B1/2 and CYP3A2 mRNAs and protein activity, as well as gene expression of glutathione *S*-transferase and NADPH-cytochrome P-450 reductase. These results clarified the inhibitory effect of PB application at a low dose, observed in our medium-term bioassay (Ito test), and indicated that the compound exhibits hormetic effects on rat hepatocarcinogenesis initiated with DEN by differentially altering cell proliferation, apoptosis and oxidative DNA damage at high and low doses.

Rat multiorgan carcinogenicity test (DMBDD model)

Several experimental in vivo bioassay systems based on the two-step carcinogenesis have been developed for the detection of carcinogenic potential of environmental chemicals. The majority of these bioassays predict carcinogenicity of test chemicals in only single organs, because the modifying effects of chemicals can be manifested only

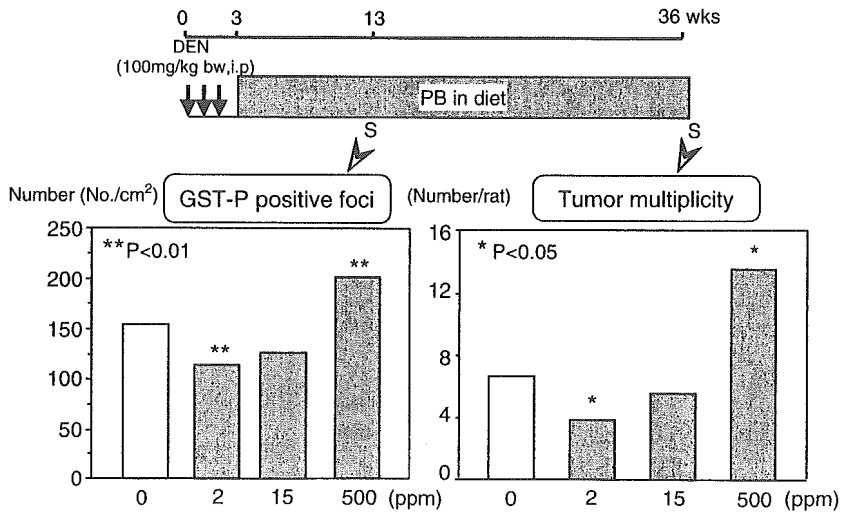

Fig. 2. Carcinogenicity of PB in the rat liver: GST-P-positive foci and tumor development.

in those organs for which appropriate initiation has been accomplished. For the purpose of developing an alternative assay approach for the detection of carcinogenicity in a variety of target organs, medium-term bioassay systems were investigated using multiorgan wide-spectrum initiation approaches. To predict the carcinogenicity of test chemicals in multiple organs and to examine their modifying potentials, the multiorgan carcinogenicity bioassay (DMBDD model) in rats was established (Imaida et al., 2003; Takahashi et al., 1992).

For clarification, the carcinogenic and modifying potential of chemicals, in the DMBDD model, male 6-week-old F344 rats were treated sequentially with 5 carcinogens (DEN 100 mg/kg b.w. in saline, i.p., *N*-methyl-*N*-nitrosourea (MNU), 20 mg/kg b.w. in citrated-buffered solution, dihydroxybutyl-di-*N*-propylnitrosamine (DHPN), 0.1% in drinking water, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), 0.05% in drinking water, 1,2-dimethylhydrazine (DMH), 40 mg/kg b.w. in saline, s.c. (DMBDD treatment) (Fig. 3). After those treatments, animals were administered 63 test chemicals for 24–32 weeks starting from week 5. Histopathological and immunohistochemical analyses of all organs/tissues were performed for the induction of preneoplastic lesions or tumors. Table 2 shows the results of chemicals tested in this assay. Seventeen out of seventeen hepatocarcinogens (100%) and 19 out of 22 non-hepatocarcinogens showed positivity in this bioassay. Five non-carcinogens were negative. For chemicals with unknown carcinogenicity, the positive rate was 47%. This bioassay appeared to be very useful for analysis of carcinogenic or modifying potential of test chemicals when their target organs are not the liver. Furthermore, this bioassay can also be useful for dose–response studies, with high sensitivity at very low doses, and can be applied in the analysis of the risk for the carcinogenic potential.

Table 2
Result of 63 test chemicals in the rat multi-organ bioassay for carcinogens

Category of chemicals	Ames test (%)			Total
	Positive	Negative	Unknown	
Hepatocarcinogen	12/12 (100)	5/5 (100)	0/0 (0)	17/17 (100)
Non-hepatocarcinogen	10/11 (91)	8/10 (80)	1/1 (100)	19/22 (86)
Non-carcinogen	0/1 (0)	0/4 (0)	0/0 (0)	0/5 (0)
Unknown	0/1 (0)	6/11 (55)	3/7 (43)	9/19 (47)
Total	22/25 (88)	19/3 (63)	4/8 (50)	45/6 (71)

In conclusion, the rat multiorgan medium-term carcinogenesis model (DMBDD model) is very useful for the investigation of the carcinogenic modifying potentials of various chemicals on tumor development.

Carcinogenicity of arsenics in animals

Arsenic is a well-documented human carcinogen and its contamination is of global concern, presenting as a significant issue in environmental health. Dimethylarsinic acid (cacodylic acid; DMA) is one of the major methylated metabolites of ingested arsenics in most mammals. In the present set of experiments, we focused on the carcinogenetic effects of DMA, using rat DMBDD models (Yamamoto et al., 1995). For initiation of carcinogenesis, animals were treated sequentially with 5 carcinogens (DMBDD treatment) as described above. After a 2-week interval, they were given 50, 100, 200, or 400 ppm DMA, respectively, in drinking water. Groups which were not given DMBDD treatment received 100 and 400 ppm DMA during weeks 6–30. In the initiated groups, DMA significantly enhanced the tumor induction in the urinary bladder, kidney, liver, and thyroid gland, with respective incidences (400 ppm DMA) being 80, 65, 65, and 45% (Fig. 4). The induction of preneoplastic lesions (GST-P-positive foci in the liver and atypical tubules

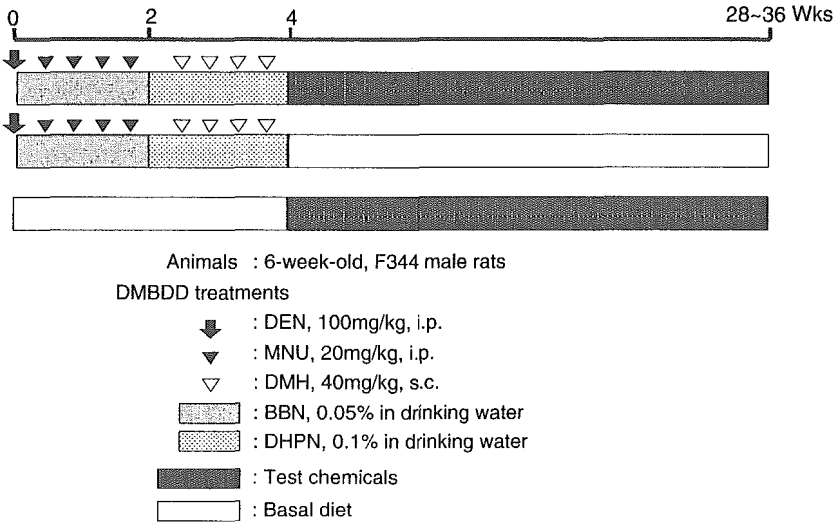

Fig. 3. Rat multiorgan bioassay for carcinogens (DMBDD test).

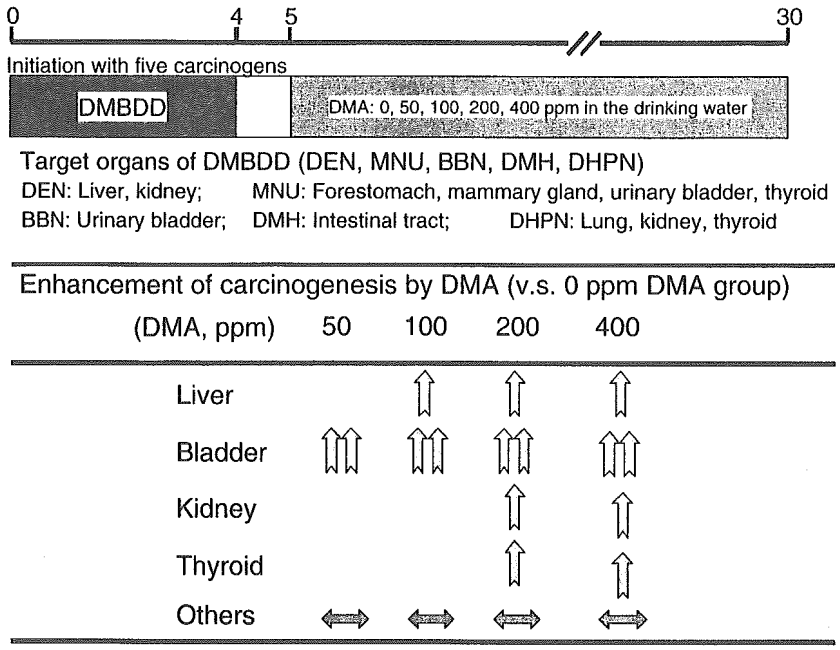

Fig. 4. Enhancement of carcinogenesis by DMA using rat multi-organ bioassay.

in the kidney) was also significantly increased in DMA-treated groups. In conclusion, DMA is acting as a carcinogen or promoter of the urinary bladder, kidney, liver, and thyroid gland carcinogenesis in rats, and this may be related to cancer induction by arsenic in humans.

To determine the carcinogenicity of DMA in male F344 rats, animals received the compound at concentrations of 0, 12.5, 50, or 200 ppm in their drinking water for 104 weeks as ordinary carcinogenicity test (Wei et al., 1999). Urinary bladder tumors were observed in 8 of 31 (26%) and 12 of 31 (39%) animals in the 50 ppm and 200 ppm groups, respectively, while none were found in the 12.5 and 0 ppm groups. No DMA treatment-related tumors were evident in other organ sites. Mutation analysis showed the DMA-induced rat urinary bladder tumors to have a low rate of H-*ras* mutations (2 of 20, 10%) and no mutations in the investigated regions of the *p53*, K-*ras* or *beta-catenin* genes. Thus, the results indicated that DMA is carcinogenic for the urinary bladder and has a promoting activity in the liver, kidney and thyroid.

In conclusion, the rat liver medium-term and multiorgan bioassay systems for carcinogens are very useful tools for the detection of not only genotoxic but also non-genotoxic carcinogens. Positive results were obtained in a relatively short period and closely correlated with the long-term carcinogenicity test. These bioassays are particularly useful and reliable methods for detecting carcinogenic or modifying potentials of low doses of carcinogens.

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REVIEW

Hormesis and dose–response-mediated mechanisms in carcinogenesis: evidence for a threshold in carcinogenicity of non-genotoxic carcinogens

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Recently the idea of hormesis, a biphasic dose–response relationship in which a chemical exerts opposite effects dependent on the dose, has attracted interest in the field of carcinogenesis. With non-genotoxic agents there is considerable experimental evidence in support of hormesis and the present review highlights current knowledge of dose–response effects. In particular, several *in vivo* studies have provided support for the idea that non-genotoxic carcinogens may inhibit hepatocarcinogenesis at low doses. Here, we survey the examples and discuss possible mechanisms of hormesis using phenobarbital, 1,1-bis (*p*-chlorophenyl)-2,2,2-trichloroethane (DDT), α-benzene hexachloride (α-BHC) and other non-genotoxins. Furthermore, the effects of low and high doses of non-genotoxic and genotoxic compounds on carcinogenesis are compared, with especial attention to differences in mechanisms of action in animals and possible application of the dose–response concept to cancer risk assessment in humans. Epigenetic processes differentially can be affected by agents that impinge on oxidative stress, DNA repair, cell proliferation, apoptosis, intracellular communication and cell signaling. Non-genotoxic carcinogens may target nuclear receptors, cause aberrant DNA methylation at the genomic level and induce post-translational modifications at the protein level, thereby impacting on the stability or activity of key regulatory proteins, including oncoproteins and tumor suppressor proteins. Genotoxic agents, in contrast, cause genetic change by directly attacking DNA and inducing mutations, in addition to temporarily modulating the gene activity. Carcinogens can elicit a variety of changes via multiple genetic and epigenetic lesions, contributing to cellular carcinogenesis.

Chemical carcinogens and human cancer

The risk of cancer in humans is dependent on environmental, occupational and recreational exposure to carcinogens as well as on spontaneous events that reflect human variation in the efficiency or fidelity of various cancer-critical processes. Assessment of carcinogenic potential of agents to which human beings are exposed is clearly of prime importance but this is complicated by the existence of both genotoxic and non-genotoxic classes of chemical carcinogens, divided on the basis of their ability to react with DNA and form adducts. It is well established that genotoxic agents can covalently bind to DNA and increase the number of mutations, thereby causing errors in DNA replication. On the other hand, errors in DNA replication themselves might cause mutations that are then inherited by progeny cells. Positive data for chromosomal effects like aneugenicity or clastogenicity, in the absence of mutagenicity, may support separate characterization of compounds that exert carcinogenic effects only at high doses (1). Non-DNA-reactive compounds, such as topoisomerase inhibitors (2,3) and inhibitors of the spindle apparatus or associated motor proteins (4–7), are considered to act by this mechanism (8).

Many chemicals that produce tumors in experimental animals have been shown to act by epigenetic mechanisms that do not necessarily involve DNA attack or hereditable genetic alteration (9). The indirect nature of the mechanisms involved means that prolonged exposure to high levels of chemicals is necessary for the production of tumors (10). With such non-genotoxic carcinogens, theoretically, cancer would not occur at exposures below a threshold at which the relevant cellular effect is not operative. Also, in contrast to DNA-reactive genotoxic effects, epigenetic mechanisms may be unique to the rodent species used for testing. Certain chemical carcinogens have been well studied and provide examples for the use of mechanistic information in risk assessment. Non-genotoxic carcinogens including tumor promoters, for example dioxin, do not bind directly to DNA but alter cell proliferation and physiology by inducing expression of enzymes involved in the xenobiotic metabolism, DNA repair, methylation and cell signaling. An altered hormonal environment may enhance the rate of cell replication by mechanisms involving receptor-mediated processes without DNA-reactivity, thus increasing the likelihood of promotion/progression of spontaneously initiated cells (11).

Threshold in carcinogenicity of environmental carcinogens

With the examination of the risk of human exposure to chemicals having carcinogenic potential, which are present in the environment, a natural question is whether a threshold exists for observed effects. Recently the concepts of 'practical' and 'perfect' thresholds for genotoxic and non-genotoxic compounds, respectively, have been proposed (8). The idea is that carcinogens can be further classified as follows: (i) genotoxic agents without a threshold in their effects; (ii) genotoxic compounds for which the existence of a threshold is possible but is

Abbreviations: α-BHC, α-benzene hexachloride; 2-AAF, 2-acetylaminofluorene; Cx32, connexin 32; DDT, 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane; DEN, diethylnitrosamine; GABA, gamma-aminobutyric acid; GST-P, glutathione S-transferase placental form; HCC, hepatocellular carcinoma; IR, ionizing radiation; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; NOEL, no-observed effect level; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; Ogg1, oxoguanine glycosylase 1; ROS, reactive oxygen species; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

not yet sufficiently supported; (iii) genotoxic carcinogens for which a 'practical' threshold is supported by studies on mechanisms and/or toxicokinetics; (iv) genotoxic carcinogens for which a 'perfect' threshold is associated with a no-observed effect level (NOEL); and (v) non-genotoxic carcinogens for which a 'perfect' threshold is associated with an NOEL (8).

Low-dose hepatocarcinogenicity of genotoxic environmental carcinogens has been recently examined as an aid to cancer risk assessment in humans and data pointing to 'practical' thresholds have been documented for 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), a food-derived hepatocarcinogen, and diethylnitrosamine (DEN). In the MeIQx case, the carcinogen was administered to male F344 rats through the diet at various doses of 0.0001–100 p.p.m. in 16 and 32 week studies (12). In a subsequent experiment it was administered to rats for 4 weeks followed by 11 weeks of phenobarbital treatment (13). In the DEN hepatocarcinogenicity study a total of 1957 F344 rats received the carcinogen at doses of 0.0001–10 p.p.m. in their drinking water continuously for 16 weeks (12). NOELs with regard to formation of glutathione S-transferase placental form (GST-P) positive foci, a preneoplastic endpoint marker lesion for carcinogenesis in the liver, were found to be 10 and 0.1 p.p.m. for MeIQx and DEN, respectively (12). Data for GST-P positive focus development with MeIQx followed by phenobarbital treatment at doses of 0.001–1 p.p.m. were similar to those with MeIQx-alone (13). Coadministration of carbon tetrachloride (14) or ethanol enhanced the induction of liver GST-P positive foci by MeIQx in each group. MeIQx–DNA adduct formation in the liver demonstated a linear relationship with all the doses tested, levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) being linearly elevated, from 1 p.p.m. MeIQx at week 4 and from 0.01 p.p.m. MeIQx at week 16. Interestingly, in a Big Blue transgenic rat mutagenesis assay, MeIQx at doses of $\leq$1 p.p.m. was found not to induce *lacI* gene mutations in the liver. This closely correlates with non-induction of GST-P positive foci (15). However, the dose of MeIQx at which *in vivo* mutagenicity was significant, was lower than that for induction of GST-P positive foci. Increase of carcinogen-DNA adducts, 8-OHdG, *in vivo* mutagenicity, induction of GST-P positive foci and lastly liver tumors appeared to be the chain of sequential events dependent on the dose of carcinogen, indicating the existence of NOELs and implying at least a 'practical' threshold for carcinogenicity of genotoxic carcinogens such as MeIQx and DEN. Data in line with these results were also obtained in low dose studies of 2-amino-1-methyl-6-phenolimidazo[4,5-*b*]pyridine carcinogenicity in the rat colon (16).

Until recently, risk assessment in the field of chemicals distinguished between two types of agents: the first comprising potentially toxic chemicals that may induce physical damage to human beings at above a certain threshold of exposure or intake (17) and the second class is believed to cause harm at any level above zero, even at very tiny doses (stochastic effects). However, the conventional view of toxicity and risk has been challenged by recent investigations pointing to potential beneficial effects of exposure to otherwise hazardous substances at very low dose levels. Most of the substances involved are non-genotoxic chemicals, acting as cytochrome P-450 inducers at high doses and exhibiting promoting effects on hepatocarcinogenesis in rodents, and the existence of a threshold was postulated for the substances acting via epigenetic mechanisms, such as phenobarbital (18,19), α-benzene hexachloride (α-BHC) (20), 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT) (21), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and caffeic acid (22). However, genotoxic carcinogens, such as 2-acetylaminofluorene (2-AAF) (23,24) and ionizing radiation (IR) (25), may also be included. Inhibitory effects of all these agents at low doses on carcinogenesis have been subsumed under the heading of hormesis (17).

The theory of hormesis

Hormesis has been defined as a dose–response relationship in which there is a biological activation at low doses but an inhibition at high doses, or vice versa, resulting in a U, J or inverted U-shaped dose–response (26). Hormetic effects have been studied for more than two decades (27) and many toxicants have shown benefits, rather than harm, with low-level exposure.

The history of hormesis originated in the laboratory of Prof. Hugo Schulz at the University of Greifswald in Northern Germany. He found that many agents appeared to stimulate metabolism at low concentrations but inhibit them at higher doses (26). This provided a toxicological explanation for his development of homeopathic ideas. As a result of the publicity following these initial studies he became the main academic hero for numerous advocates of homeopathy, and thus the theory of hormesis was born in close association with homeopathy as a preventive/therapeutic modality (26). Interest in the effects of low doses rapidly expanded, especially with many studies of interactions involving (mainly) plants, bacteria and fungi, most notably in Europe, USA and Japan (26). Hormetic effects were observed at low exposure levels based on the dose–response pattern with data from developmental toxicity studies, indicating that there might actually be a reduced risk of toxic effects at low exposure levels (28). Hormesis implies the existence of a threshold dose level and there are dose–response models that include parameters that account for the threshold.

With IR, hormesis was interpreted to be due to adaptation to background radiation exposure, as well as metabolic protection against the array of other abiotic stresses in the environment (25,29). Weak endogenous carcinogens, such as reactive oxygen species (ROS), as well as micronutrient deficiencies and environmental toxins are obvious causes of non-radiation induced DNA damage, which might lead to oncogenic transformation in non-irradiated cells (30). The results suggested that at the level of background radiation various forms of non-radiation DNA damage in tissues occur to much higher extents than those due to the low-dose radiation exposure. It has been proposed from the published data that mammalian cells have the physiological capacity to protect themselves constantly by preventing and repairing DNA damage. Furthermore, damaged cells are susceptible to removal by apoptosis or the immune system. Low-dose radiation was suggested to induce cellular signaling that may stimulate cellular protection systems over hours to weeks. Enhanced and persistent protective responses might reduce the steady-state level of non-radiation DNA damage, thereby impacting on deleterious outcomes such as cancer and aging (30).

Hormesis in carcinogenesis

The question whether the concept of hormesis can be generalized to carcinogenesis has been recently discussed by

E.Calabrese and L.A.Baldwin (31,32). They cite numerous examples in well-designed studies providing evidence for U- and J-shape dose relationships with respect to different biomarkers of carcinogenesis in different animal models. For some chemicals tested, carcinogens were found to be similar to other toxicants in improving the outcome at low doses, although the mechanisms of their action remained unclear. Therefore, it appears very important to answer the question of how carcinogens act at very low doses. Early stage carcinogenesis includes initiation with the occurrence of DNA damage and adaptative DNA repair. In 1983, Camurri *et al.* (33) observed a decrease of chromosomal aberrations with low-dose styrene treatment. The response of human keratinocytes to a low dose of the well-known methylating agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, was studied by Kleczkowska and Althaus (34). It was found that at concentrations in the 0.05–50 nM range DNA unwinding and DNA strand breaks were significantly reduced, while at high doses they were enhanced compared with the control case. Inhibition activity regarding DNA damage at low doses was explained by activation of poly(ADP)-ribose. Furthermore, assessment of the effects of Hg^{2+} on O^6-methylguanine-DNA methyltransferase activity of human buccal fibroblasts by Liu *et al.* (35) revealed elevation at low doses of 0.3–3 μM. In the dose–response curves of rat hepatic DNA damage for different types of carcinogens assessed by Kitchen and Brown (36), 11 showed non-monotonic character with some treated values lower than in controls.

The promotion stage of carcinogenesis has also been studied in the low dose range with regard to various parameters of interest. Examples include cell turnover with caffeic acid in the rat forestomach and kidney, altered hepatic foci formation with TCDD in DEN-pretreated partially hepatechtomized rats (22) and urinary bladder hyperplasia in saccharin-treated rats (37). Several chronic bioassays for carcinogenicity in rats and mice have demonstrated a negative correlation between proliferative hepatocellular lesions and lymphomas at low and medium dose levels (38). In addition, TCDD at hepatocarcinogenic doses was reported to be capable of causing dose-dependent reduction in mammary and uterine tumors (39). In 1994, Cook (40) reported that dioxin-treated rats displayed substantial decrease in tumors of the adrenals and pancreas and more modestly, in the liver. Examples of hormesis also include TCDD-mediated reduction in tumor incidence after exposure to low doses of radiation (25) or metals such as selenium (41). U-shape responses were also observed for chemically induced pulmonary tumors (42–44) and testicular cancer (45).

Hormesis in phenobarbital hepatocarcinogenicity

Recently, especial attention has been devoted to the carcinogenicity of low doses of phenobarbital, a sedative and anticonvulsant, which is used widely for long-term clinical therapy. It is also a well-known non-genotoxic carcinogen and tumor promoter in rodents. Epidemiological studies have not shown phenobarbital-related tumors in humans, indicating that humans may have low sensitivity to toxic effects of phenobarbital. In the rat, Goldsworthy, *et al.* (46) reported no promotion by phenobarbital <10 p.p.m. with regard to the enzyme-altered foci. Furthermore, Kitagawa (47) found inhibitory effects of both phenobarbital and another tumor promoter, DDT, on carcinogenesis when given together with relatively high doses of carcinogens. Similarly, Pitot *et al.* (48) found a slight decrease of altered hepatic foci by 10 p.p.m. phenobarbital and Maekawa *et al.* (49) demonstrated similar results with 1 p.p.m. phenobarbital. To determine the practical threshold level for hepato-promoting effects of phenobarbital, Kitano *et al.* (18) investigated dose dependence using a rat liver medium-term bioassay (Ito test) (50). When phenobarbital was administered to rats in a wide range of doses of 0.01–500 p.p.m. in the diet for 6 weeks after a single intraperitoneal injection of DEN in serial experiments, GST-P positive foci were found to be increased dose dependently in rats that were given 60–500 p.p.m. However, with doses in the range of 1–7.5 p.p.m., decrease was evident as compared with the control group, this being statistically significant at 1 and 2 p.p.m. (Figure 1). It was concluded that phenobarbital effects reflect hormesis in the rat liver, indicating the existence of a threshold for its carcinogenicity, suggested to be related to the suppression of cytochrome P-450 CYP3A2 protein expression by low doses of the chemical (18).

For further clarification of the hormetic influence of phenobarbital, Kinoshita *et al.* (19) investigated doses of 0, 2, 15 and 500 p.p.m. applied in diet to male F344 rats for 10 or 33 weeks after initiation of hepatocarcinogenesis using DEN. Formation of GST-P positive foci and liver tumors was inhibited at 2 p.p.m. after 10 and 33 weeks of phenobarbital administration, respectively (Figure 2). Histopathological examination further demonstrated a significant reduction in the multiplicity of total tumors, in particular, hepatocellular carcinomas (HCCs), and a tendency for decreased incidences of HCCs and adenomas at 2 p.p.m. (19). In contrast, a high-dose administration resulted in strong elevation of HCC and total tumor multiplicities, this appearing to be related to increased generation of hydroxyl radicals, a marker of oxidative damage 8-OHdG, CYP2B1/2 and CYP3A2 mRNAs and the protein level, and activity and gene expression of other Phase I and II xenobiotic metabolizing enzymes. Inhibition at low doses was considered to be due to the suppression of 8-OHdG generation and cellular proliferation within areas of GST-P positive foci, as well as programmed cell death, apoptosis, in background liver parenchyma. The decrease of 8-OHdG levels induced by phenobarbital at low dose was possibly a result of elevated expression of the gene encoding the enzyme oxoguanine glycosylase 1 (Ogg1), which is responsible for the repair of 8-OHdG lesions. The reduction of apoptosis in the normal-appearing liver tissue surrounding the GST-P positive foci, which might have been due to the inhibition of oxidative DNA damage, was suggested to suppress enlargement of foci because of elevated sensitivity to stimuli for regeneration (19). Another explanation for the suppressive effect of phenobarbital on the development of preneoplastic lesions might involve stimulation of hepatic drug-metabolizing enzymes, which detoxify carcinogens (48). Activation of P-450 isoenzymes CYP2C11 and NADPH-cytochrome P-450 reductase (OR) in liver microsomes observed after the administration of phenobarbital at a low dose, if not accompanied by elevation of their protein expression leading to the generation of large amount of ·OH, might have a protective effect (19). The available results thus indicate that the compound exhibits hormetic effects on rat hepatocarcinogenesis initiated using DEN by differentially altering cell proliferation, apoptosis and oxidative DNA damage at high and low doses.

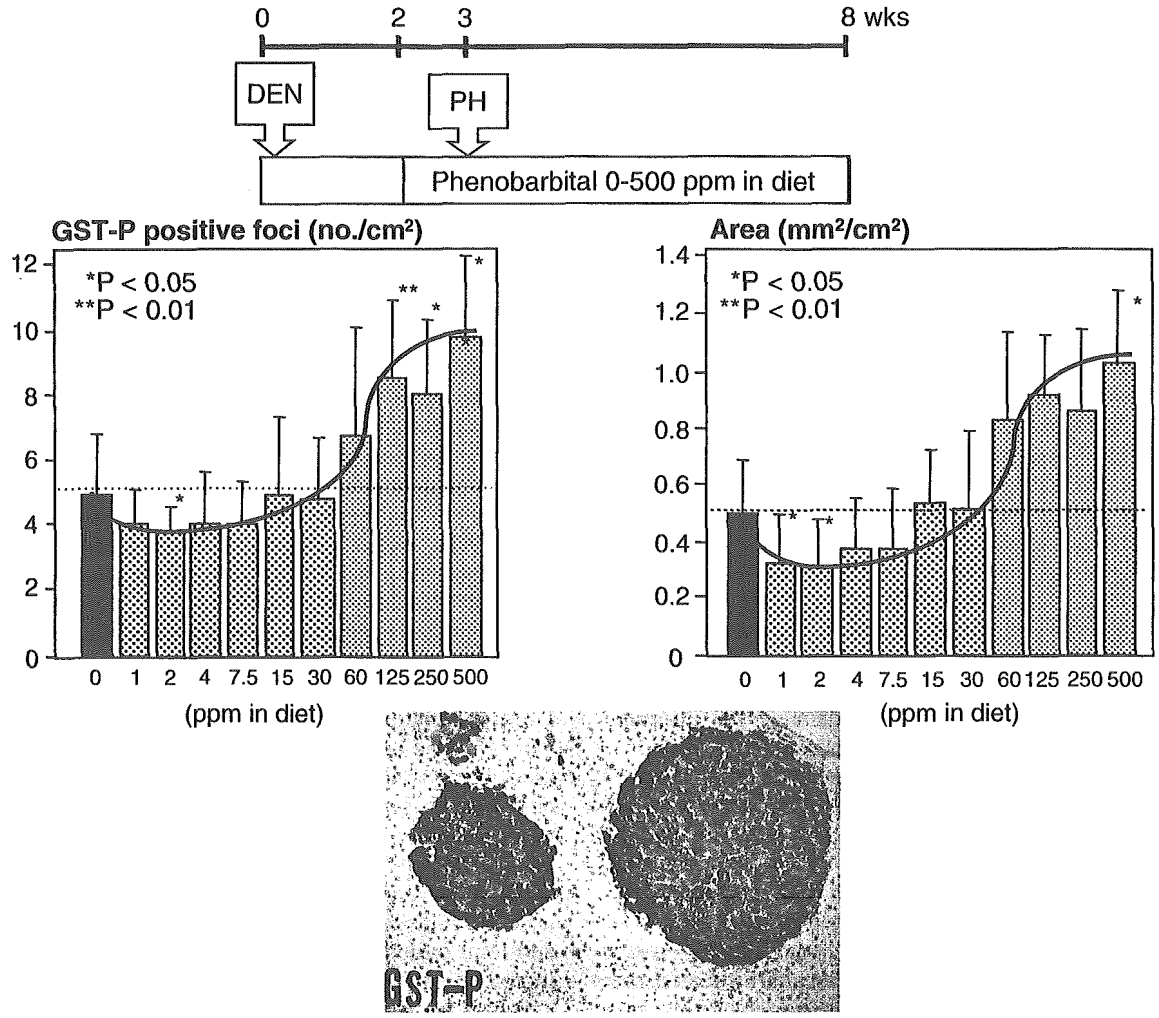

Fig. 1. Induction of GST-P positive foci in the livers of rats treated with phenobarbital in a medium-term bioassay (Ito test). PH, 2/3 partial hepatectomy.

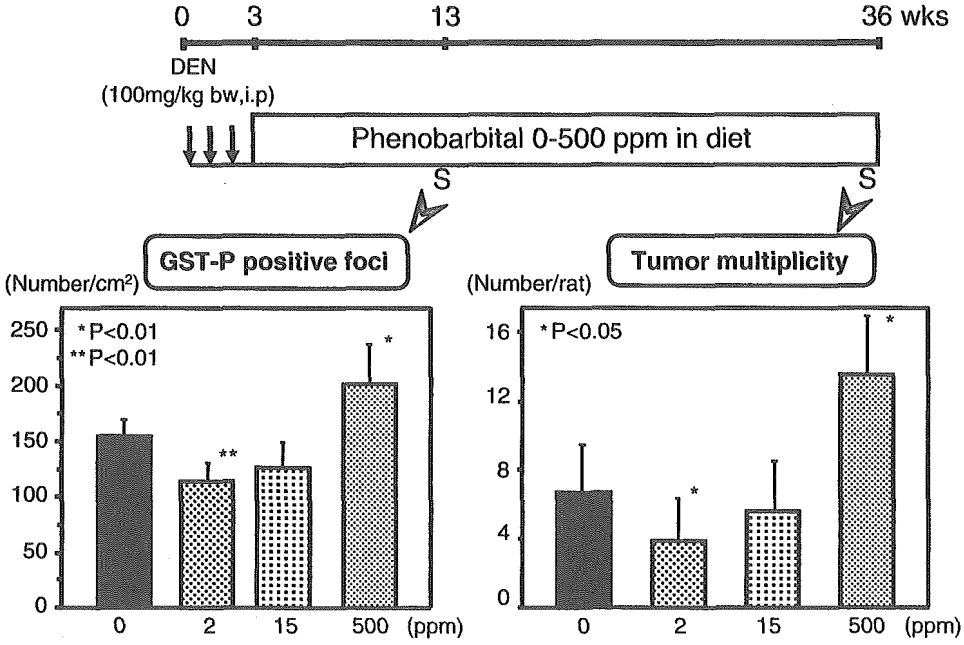

Fig. 2. Hepatocarcinogenicity of phenobarbital in the rat liver: GST-P positive foci and tumor development (DEN→PB). See online Supplementary material for a color version of this figure.

Dose–response for α-benzene hexachloride hepatocarcinogenicity

α-BHC, a major organochlorine byproduct in the manufacture of lindane (γ-BHC), has been used in admixtures with lindane for agricultural purposes. Among the eight isomers of BHC, the α-isomer has been categorized as a non-genotoxic carcinogen as it induces liver tumors in rodents after high-dose administration in the long-term, but no mutagenicity is shown in the Ames test. The major metabolite in α-BHC metabolism by the cytochrome P-450 oxidoreductase system is 2,4,6-trichlorophenol. After dechlorination and dehydrochlorination of α-BHC, removable chlorine atoms might react with hydrogen peroxide to produce hypochlorous radicals binding to DNA and formation of chlorinated DNA adducts, like 8-chloro-2-deoxyguanosine, 5-chloro-2-deoxycytidine and 8-chloro-2-deoxyadenosine (51,52). Long-term treatment with high doses of α-BHC (such as 500 or 1000 p.p.m.), but not β- and γ-BHC, has been found to induce hyperplastic nodules and carcinomas in the livers of rats and mice (53,54). Early toxicological studies revealed that α-, β- and γ-BHC are potent inducers of hepatic monooxygenases in rats (55), in addition to causing liver enlargement (56,57). Since induction of the monooxygenase system is assumed to influence the promotion stage (58,59), the mechanism of α-BHC carcinogenicity is likely to be due to its influence on spontaneously initiated hepatocytes (53,54).

To investigate whether α-BHC exhibits hormesis with respect to its hepatocarcinogenesis the dose dependence of its promoting effects was first investigated by Masuda *et al.* (20) in a medium-term rat liver bioassay (Ito test). When F344 male rats were given α-BHC at a wide range of doses from 0.01 to 500 p.p.m. in the diet for 6 weeks after a single intraperitoneal injection of DEN, quantitative values for numbers and areas of GST-P positive foci were dose-dependently increased at 0.5–500 p.p.m. However, a tendency for a decrease was observed with 0.01 and 0.1 p.p.m. α-BHC (Figure 3). As observed with phenobarbital, CYP3A2 protein levels and activities showed a good correlation with the numbers and areas of GST-P positive foci. This experiment provided supportive evidence for hormesis in the promotion of rat hepatocarcinogenesis by α-BHC and suggested that the mechanism might be related to the suppression of P-450 isoenzyme CYP3A2 protein expression by low doses (20).

A second study was conducted with α-BHC applied to F344 rats at doses of 0.01–500 p.p.m. for 10 weeks after DEN initiation (unpublished data). While α-BHC promoted the formation of GST-P positive foci at the dose of 500 p.p.m., both the numbers and areas of preneoplastic lesions were found to be significantly reduced with 0.05 p.p.m. The dose–response curves for cytochrome P-450 content, NADPH-cytochrome P-450 reductase activity and 8-OHdG formation exhibited essentially the same patterns as for GST-P positive foci. A low dose of α-BHC also tended to upregulate Ogg1 mRNA expression. Similar to the phenobarbital case, α-BHC treatment led to increase in PCNA positive cells within the areas of GST-P positive foci at a dose of 500 p.p.m. but gave decreased values at low doses. Though the response curves for CYP2B1 and 3A2 catalytic activity, protein levels and mRNA expression showed thresholds, CYP2C11 activity exhibited an inverted J-shape. This major constitutive male-specific isoform was thus found to be upregulated by a low dose of α-BHC treatment at the transcriptional level and with regard to catalytic activity detected with 2α- and 16α-testosterone metabolites. Thus, CYP2C11 might take part in detoxification while CYP2B1 and 3A2 isoenzymes are considered to participate in bioactivation of α-BHC and increase its toxicity, given the correlation with GST-P positive foci and oxidative DNA damage. The non-linear threshold dose–response observed at low doses with respect of CYP2B1 and 3A2 can be deemed to be a result of a multi-step process 'turning on' orphan nuclear receptors, constitutive androstane receptors and the pregnane X receptor, which is known to regulate CYP2B1 and 3A2 transcription by binding as a heterodimer

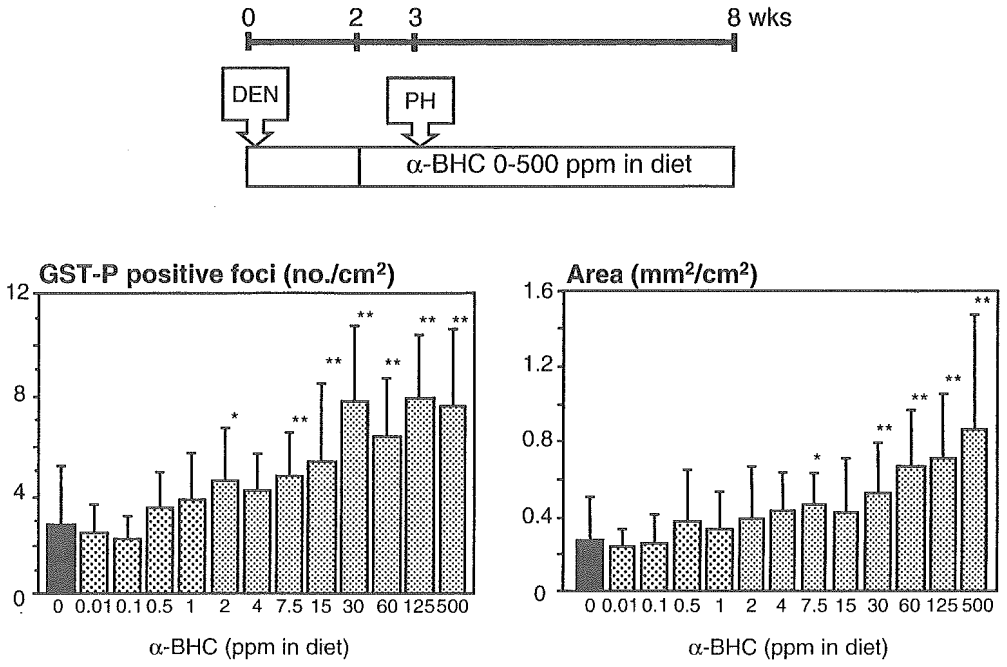

Fig. 3. Induction of GST-P positive foci in the liver of rats treated with α-BHC in a medium-term bioassay. PH, 2/3 partial hepatectomy.

to the retinoid X receptor, RXR (60,61). Furthermore, in the same study it was shown that glutathione S-transferase, which plays an important role in detoxifying α-BHC, demonstrates a threshold in its activity towards α-BHC at low doses (62, unpublished data).

The possibility of a hormetic effect of α-BHC regarding formation of liver tumors *in vivo* was further examined in F344 rats at doses from 0.01 to 500 p.p.m. given in the diet for 36 weeks after initiation of hepatocarcinogenesis using DEN (unpublished data). Incidences and multiplicities of liver tumors were found increased in a dose-dependent manner by α-BHC at doses of 0.5–500 p.p.m., while a tendency for decrease in their values was found in the low-dose 0.01 and 0.1 p.p.m. groups, similar to the case with rat liver preneoplastic lesions (unpublished data).

From these results it was concluded that α-BHC exhibits hormesis with regard to its hepatocarcinogenicity at low dose by mechanisms involving induction of detoxifying enzymes, as well as by influencing free radical production and oxidative stress, and consequently bringing pathological change in the liver. In these studies, the dose–response relationship for GST-P positive foci was represented using a J-shape curve, in line with the previous investigation of this chemical using the Ito test (20).

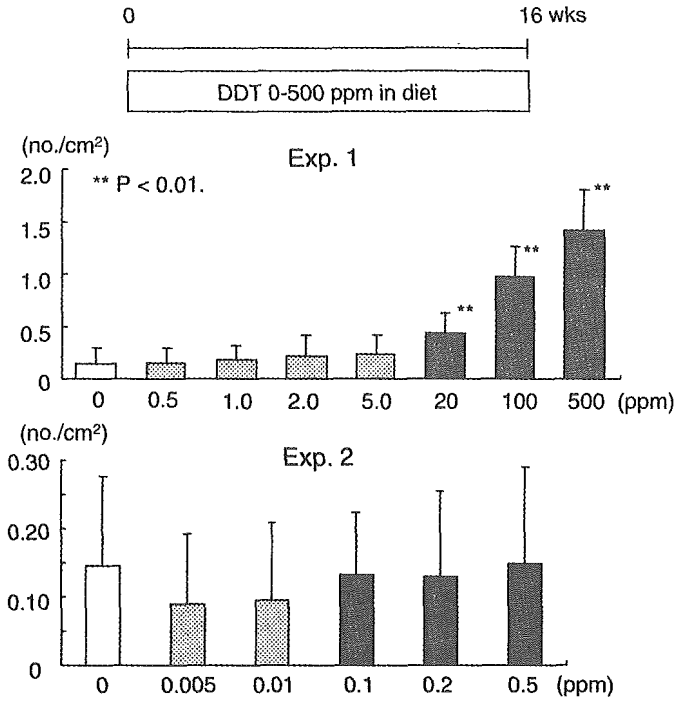

Fig. 4. Induction of GST-P positive foci in the livers of rats treated with DDT for 16 weeks.

Possibility of a hormesis for DDT in hepatocarcinogenesis

Inhibitory effects on the induction of GST-P positive foci were also noted with low doses of another non-genotoxic carcinogen, DDT (21). First, in the study of Sukata *et al.* (21), F344 rats, 21-day-old at the commencement, were administered DDT at doses from 0.005 to 500 p.p.m. in their diet for 16 weeks. In another experiment Kushida *et al.* (63) investigated the possibility of hormesis after DDT administration to F344 rats for 11 and 43 weeks following initiation of hepatocarcinogenesis using DEN. In both experiments the doses of ≥20 p.p.m. were associated with dose-dependent induction of GST-P positive foci in the liver. In contrast, 0.005 and 0.01 p.p.m. administration resulted in a tendency for decrease in values below the control level (Figure 4). Histopathological analysis of liver nodules also revealed a tendency for decrease in the incidence and multiplicity of HCCs in the low-dose groups as compared with the DEN initiation controls. The multiplicity of total tumors also tended to decrease, although incidences were similar. Alteration of the GST-P positive foci in the low-dose groups was correlated with a tendency for decrease in the CYP3A2 protein level as well as induction of IL-1 receptor type I (IL-IRI) and TNF-α receptor type I, whose ligands have roles in downregulating CYP3A2 and influencing cellular proliferation or apoptosis (21). IL-1R1 is known to be a cell surface molecule involved in cell signaling (64), while IL-1 inhibits regeneration of rat liver cells (65) and tumor cell growth (66), and inhibitory actions of IL-1β on hepatocyte DNA synthesis are effected by *iNOS* gene expression and NO production under IL-1R1 control (67).

It was found that within GST-P positive areas, cell proliferation was slightly lower in the 0.005 p.p.m. DDT dose group than in the only DEN treated group (21). As observed in experiments with phenobarbital and α-BHC, CYP2B1/2 and CYP3A2 protein levels in the liver microsomal fraction were significantly elevated by high doses of DDT. In line with previous results, 8-OHdG formation was significantly suppressed by a low dose of the chemical, presumably related to effective DNA repair and co-repair of endogenous damage, which may exceed formation of adducts (68). Oxidative stress in the low-dose group was suggested to be decreased because of the lowered CYP3A2 expression and formation of 8-OHdG balanced through elimination by Ogg1 (21,63). Furthermore, in the low DDT dose group, mRNA expression and immunohistochemical staining of connexin 32 (Cx32) were found to be elevated (21). Many previous studies indicated that high doses of DDT and other non-genotoxic carcinogens inhibit Cx32, resulting in the loss of the function of gap junction intracellular communication (GJIC) (60–71) and release of potentially initiated cells from growth constraints imposed by normal neighboring cells, resulting in clonal expansion and ultimately tumor formation and progression (71,72). In the present study, mRNA expression of one of the transcriptional factors, HNF-1α, which regulates Cx32 expression (73,74), was in good correlation with that of Cx32 (62). Differential alteration of HNF-1α is suggested to be one of the possible mechanisms by which DDT might inhibit or promote rat hepatocarcinogenesis.

Hormetic effects observed with ethanol

Effects of alcohol intake on cardiovascular diseases (75), stroke (76), all causes of death (75,77) and cancer mortality (78) are known to demonstrate U- or J-shaped curves; that is, those who consume very less alcohol have the lowest risk. The relationship between smoking dose or drinking dose and risk for stomach cancer has also attracted great interest as to whether strict dose-dependence or a U-shaped curve might be evident (79). Recently, the risk of stomach cancer was reported to increase linearly with the smoking dose, but not with the drinking dose. Kikuchi *et al.* (80) showed that light drinkers in Japan have the lowest risk of developing stomach cancer among both male and female subjects, and heavy drinkers

the highest risk among males, the association being J-shaped among male subjects and U-shaped among female subjects, and thus very similar to the association with risk of cardiovascular diseases and stroke. J- or U-shaped dose–response curves were suggested to offer an explanation for the fact that more studies on stomach cancer have demonstrated an association with smoking than with drinking (80).

In a recent study the promoting effects of ethanol at different doses on MeIQx induced liver carcinogenesis in F344 rats was evaluated (unpublished data). While a high dose of ethanol (10–20% in drinking water) was found to exert clear promotion of development of MeIQx induced liver cancer in rats, no significant inhibitory activity on hepatocarcinogenesis was observed after the administration of ethanol at low doses (0.1–1%).

Adaptive mechanisms

To explain hormetic effects, adaptive responses have been proposed. When experimental animals are exposed to biologically effective levels of chemicals, their bodies have to deal with chemical perturbation and diverse responses are elicited. For some chemicals, the initial response constitutes an adaptive effect that maintains homeostasis (24,26). Disruption of this balance at any level of organization may lead to an adverse effect, or toxicity. When target cells are exposed to non-genotoxic carcinogens, as described above, it is to be expected that machinery to conserve homeostasis would be switched on, for detoxification and excretion, with preservation of the cell cycle and programmed cell death regulation through cell signaling. At very low doses of chemicals, such mechanisms in target cells might more than compensate for cell injury so that not only a dose threshold but also a reduction in lesion development, as compared with the control case, may occur. This would explain the U- or J-shaped response curves obtained for phenobarbital, α-BHC and DDT hepatocarcinogenicity (Figure 5).

Hepatic adaptive responses usually involve actions of the chemical on cellular signaling pathways, which is often receptor mediated, leading to changes in gene expression and ultimately alteration of the 'metabolome', directed toward maintaining homeostasis through modulation of various cellular and extracellular functions. At all levels of organization, adaptive responses are beneficial in that they enhance the capacity of all units to respond to chemical induced stress,

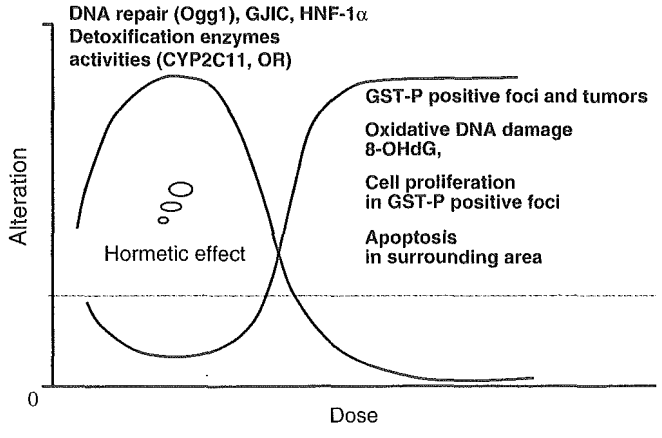

Fig. 5. Potential mechanisms mediating hormesis in carcinogenesis.

are reversible and preserve viability. In contrast, adverse or toxic effects produced by genotoxic chemicals often involve chemical reactions with cellular macromolecules such as DNA or proteins and result in disruption of homeostasis. Such effects can be non-reversible at all levels of organization resulting in mutations or inactive protein molecules. Examples of compounds eliciting adaptive effects are provided by phenobarbital and ciprofibrate, whereas *p*-dichlorobenzene and 2-AAF, for instance, exhibit primarily toxic effects.

Bystander effects

Numerous investigations have revealed that several cancer relevant effects of IR can occur in cells that have received only cytoplasmic or plasmalemmal membrane exposure to IR (81–88). Furthermore, many effects that have been attributed to IR-induced damage to nuclear DNA or that occur following irradiation of the cytoplasmic compartment of cells can also occur in cells that have received no direct exposure to IR. These so-called bystander effects as well as adaptive responses are linked to biological effects of radiation and chemical treatments and involve intracellular communication systems (both gap junctional and extracellular communication) (81). Bystander effects are considered to be induced by radiation in non-irradiated cells when an extracellular signal produced by a radiation-targeted cell is received by a non-hit cell, or by gap junctional direct transfer of some radiation-induced signals (82). Bystander effects may include increase in intracellular ROS, induction of mutations, enhanced cell growth, apoptosis, genomic instability and neoplastic transformation, as well as cell death (83–88). Both direct transfer of small molecules or ions through gap junctions and extracellular signaling by secreted factors (hormones, cytokines, growth regulators, etc.) maintain homeostasis and might be related to hormesis (82). The implications of bystander effects of low- and high-dose radiation exposure for potential health endpoints still need to be resolved.

Hormetic effects with endogenous ROS

Exposure to different chemical carcinogens for which hormetic effects are proposed leads to formation of ROS, and frequently to induction of cytochrome P-450 species, with induction of oxidative stress. ROS are genotoxic in principle, and the question arises as to whether chemicals that increase ROS production will add to an endogenously produced background level of DNA lesions, or whether compensatory mechanisms exist that may result in non-linear dose effects. Endogenous ROS cause detectable background levels of DNA damage, namely in the form of oxidized bases (e.g. 8-OHdG), apurinic (AP) sites and strand breaks. Oxygen radicals also attack other cellular components such as lipids to generate reactive intermediates that couple to DNA and give rise to exocyclic etheno- and propane-adducts, and 1,N^6-ethenodeoxyguanosine and 3,N^4-ethenodeoxycytidine (89–91). Such adducts will have mutation-associated consequences upon cell replication (92). The continuous production of free radicals from radiation and other sources has stimulated organisms to evolve repair systems for oxidative base modifications or chromosome breaks. Alteration to DNA molecules triggers repair, and frequent activation may increase the general repair capacity, irrespective of the cause of the damage. Repeated exposure to ROS may thus lead to an

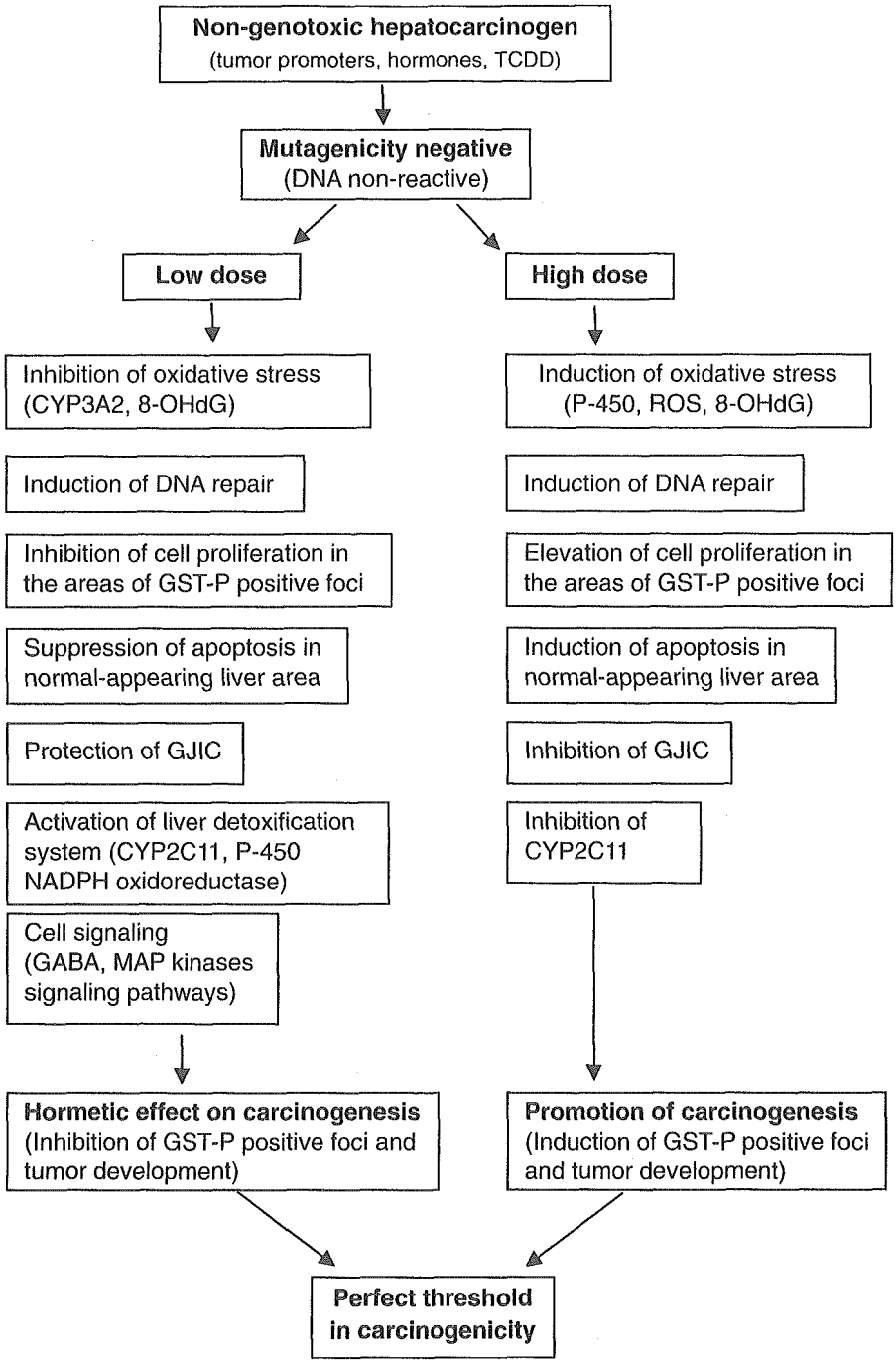

Fig. 6. Proposal of a flow scheme toward dose–effect relations, risk assessment and mechanisms of action of non-genotoxic chemical carcinogens.

adaptive response, mitigating the mutagenicity of oxidative DNA lesions. DNA repair is a crucial factor in maintaining a low steady-state level of DNA damage and its impairment is implicated in processes that promote human cancer (93). It is difficult to state at the present time the precise role of ROS-induced DNA damage in carcinogenesis and how genetic and epigenetic events induced by ROS interact with cell transformation and malignant progression. However, many aspects have already been elucidated, indicating that at low levels of ROS, adaptive responses, repair and antioxidative defenses are strengthened, whereas at high levels they may be overwhelmed. Whether or not the induction of a detoxifying enzyme qualifies as a basis for a practical threshold depends on the speed and capacity of removal of the reactive species from the system compared with the speed of the translocation of the reactive species from the site of its generation to the nucleus and reaction with the DNA.

Alteration to cell proliferation, apoptosis and DNA repair

Induction of ROS has been observed to alter cell proliferation and apoptosis in the tissues. While marked increase in oxygen radicals in the rat liver in cases of non-genotoxic carcinogens, for example phenobarbital, α-BHC and DDT at high dose, leads to elevation of PCNA indices in areas of GST-P positive foci. Cell proliferation rates at low doses were found to be

decreased (19). Suppression of liver nuclear DNA 8-OHdG formation at low dose may be associated with reduction of cell proliferation within GST-P positive foci. Furthermore, apoptosis, significantly induced by high-dose administration in liver tissue surrounding GST-P foci was suppressed in the low dose groups, with strong similarity to the pattern observed for 8-OHdG. Apoptosis of normal-appearing liver tissue has been proposed as one factor regulating the size of foci, as enlargement of GST-P positive foci presumably requires regenerative stimuli. In a low-dose phenobarbital study, the results of cDNA microarray analysis indicated 2 p.p.m. of phenobarbital to specifically enhance mRNA expression for glutamic acid decarboxylase (GAD65), an enzyme involved in the synthesis of gamma-aminobutyric acid (GABA), while suppressing expression of MAP kinase p38, JNK1, 2 and other intracellular kinases (19). Recently, a negative correlation between the expression of GABA-A receptors in hepatocytes and thymidine incorporation in liver specimens was reported, albeit without evidence of a causal relationship, and the GABA-B receptor subtype is known to be involved in hepatocyte DNA synthesis and mediation of growth stimulation (94,95). Thus, the suppression of gene expression of signal transduction modulators, such as MAP kinase p38, JNK1 and 2, and other intracellular kinases, might be a factor related to the inhibitory effect of phenobarbital on cell proliferation.

The fact that DNA repair protects cells from fixation of DNA damage in the newly synthesized DNA strand as heritable mutations means that outcome of exposure to carcinogens is dependent on the race between repair and proliferation-dependent DNA synthesis. The combination of elevated repair and decreased cell division may more than compensate for deleterious influence. Application of higher doses of the same substance may result in an increased tumor incidence because of cell cycle progression due to cytotoxicity and regenerative cell proliferation. As a consequence, a J-shaped dose–effect curve results. It is proposed that cell cycle progression and regenerative proliferation represent the key parameters concerning threshold mechanisms, although apoptosis also contributes to this. This would be particularly important for epigenetic carcinogens, whereas the genotoxic substance levels of DNA damage in target tissues are far higher. Of high interest are genotoxic substances like MeIQx or DEN for which carcinogenicity, induction of regenerative proliferation and genotoxicity also appear to act through processes with a threshold. Furthermore, it should be borne in mind that apoptosis and the control of neoplastically transformed cells by the immune system may be additional factors influencing the shape of the dose–effect curve.

Conclusions

In summary, recent data on the effects of non-genotoxic carcinogens indicate the existence of hormesis and a 'perfect' threshold for carcinogenicity (Figure 6). Hormesis by non-genotoxic carcinogens implies the maintenance of homeostasis, with adaptive responses involving cell proliferation and apoptosis, DNA damage and repair, cell signaling, and cell–cell communication. The findings have broad implications for cancer risk assessment methods, experimental design and the establishment of optimal drug doses, taking advantage of adaptive effects. Quantitative analyses based on biological models are necessary, with attention to factors that affect the degree of non-monotonicity. Further analyses along these lines should promote scientific discussion of biphasic dose–response curves and the concepts of 'hormesis' and thresholds, particularly for tumor induction by non-genotoxic carcinogens.

Supplementary material

Supplementary material can be found at: http://carcin.oxfordjournals.org/

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Three GnRH receptor types in laser-captured single cells of the cichlid pituitary display cellular and functional heterogeneity

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The role of multiple gonadotropin-releasing hormone receptor (GnRH-R) types in the regulation of gonadotropic and nongonadotropic cells remains speculative. To address this issue, we developed a technology integrating laser-captured microdissection of single digoxigenin-labeled pituitary cells coupled with real-time quantitative PCR to examine the expression profiles of three endogenous GnRH-R types (R1, R2, and R3) in immature and mature males of tilapia *Oreochromis niloticus*. Here, in addition to gonadotropes (luteinizing and follicle-stimulating hormone, FSH), we show GnRH-Rs are also present in lactotropes, somatotropes, thyrotropes, melanotropes (melanocyte-stimulating hormone, MSH), corticotropes and somatolactin cells. Subpopulations of pituitary cells express single (42.9%), multiple (32.4%) or lack (24.7%) GnRH-Rs. In immature males, the percentage of FSH cells containing combinations of GnRH-Rs was significantly higher (R1+R2: 24%, $P < 0.05$; R1+R2+R3: 25%, $P < 0.01$) than in mature males, whereas the percentage showing only R1 and R1 and R3 transcripts ($P < 0.05$) was higher in mature males. Significantly greater copies of R1 and R3 transcripts were found in MSH cells of immature and mature males, respectively ($P < 0.05$). GnRH-R transcripts in other pituitary cells (lactotropes, R1 and R2; somatolactin cells/thyrotropes/corticotropes, R1, R2, and R3) were significantly higher in mature males ($P < 0.05$) but were unaltered in somatotropes and luteinizing hormone cells. Thus, FSH and MSH cells are required for both reproductive states, whereas other pituitary cells are recruited only during testicular maturation. The differential expression of GnRH-Rs in gonadotropic and nongonadotropic cells demonstrates cellular and functional heterogeneity of mechanisms controlling normal sexual development.

G protein | *in situ* hybridization | tilapia

Gonadotropin-releasing hormone (GnRH) is now recognized as a family of 16 multifunctional neuropeptides in vertebrates (1–3). It is well documented that all vertebrate species ranging from fish to humans possess two (hypothalamus, GnRH1; midbrain, GnRH2) or, as in recently derived teleosts, three GnRH types (caudal olfactory bulbs, GnRH3) (1–3). GnRH1, GnRH2, and GnRH3, in addition to stimulating gonadotropes (follicle-stimulating hormone, FSH; luteinizing hormone, LH), are potent regulators of somatotropes [growth hormone (GH) cells], lactotropes [prolactin (PRL) cells], and somatolactin (SL)-containing cells in teleosts (4–9). Because GnRH exerts its actions through binding to GnRH receptors (GnRH-Rs) (10), it is, therefore, conceivable that the three GnRH types have their respective cognate receptors expressed in different pituitary cells. Multiple GnRH-Rs have been cloned (10) and their transcripts identified in LH and GH cells (11), and GnRH-R proteins in LH, GH, and PRL cells (4) of teleosts. However, the distribution of GnRH-R transcripts in other endocrine cell types of the adenohypophysis has never been evaluated. Besides, it is unknown whether transcripts of multiple GnRH-R types are coexpressed in individual pituitary cells. Therefore, there is the need to precisely identify individual cells in the pituitary that express GnRH-Rs to formulate possible roles of GnRHs and their cognate receptors.

We have successfully cloned three GnRH-R types from the brain and pituitary of the tilapia *Oreochromis niloticus* [GenBank accession nos.: AB111356 (GnRHR1), AB111357 (GnRHR2), and AB158490 (GnRHR3); unpublished data]. Furthermore, we developed a technology integrating laser-captured microdissection (LCM) of single digoxigenin (DIG)-labeled pituitary cells coupled with real-time quantitative RT-PCR (RT-Q-RT-PCR) that allows harvesting identified individual pituitary cells with precision and high preservation of mRNA for analysis. Thus, to increase understanding of the role of GnRH types on the pituitary–gonadal axis, the present study was designed to analyze the effects of sexual maturity on the expression profiles and the functional states of the three GnRH-R types in single gonadotropic and nongonadotropic cells. For this purpose, we used immature and mature males of tilapia because sexual maturity in males is marked by the development of pinkish-red coloration, initiation of nest-building behavior, and increase aggressiveness, and there is evidence in males that shows GnRH1 is important for sexual maturation, whereas GnRH2 and GnRH3 might have roles in reproductive behaviors or nonreproductive functions (12).

Materials and Methods

Experimental procedures in the present study were performed under the guidelines of the Animal Care Committee of Nippon Medical School, Tokyo. Male tilapia *O. niloticus*, maintained in fresh water at 27 ± 1°C with a natural photo regime (10/14-h light/dark cycle), were used in the present study.

Tissue Preparation and *in Situ* Hybridization for Pituitary Hormones. Immature [standard length, 5.25 ± 0.24 cm; body weight, 4.77 ± 0.51 g; gonadosomatic index (GSI), 0.032 ± 0.018; $n = 5$] and mature (standard length, 11.13 ± 0.68 cm; body weight, 45.73 ± 7.02 g; GSI, 1.28 ± 0.39; $n = 5$) males were anesthetized by immersing in a 0.01% solution of 3-aminobenzonic acid ethyl ester (MS222; Sigma) before they were killed by decapitation. The brains with pituitaries attached were dissected and fixed in 4% buffered paraformaldehyde for 6 h at room temperature, cryoprotected in 20% sucrose, and embedded in Tissue Tek OCT compound (Sakura Finetechnical, Tokyo). Pituitary sections were cut in sagittal planes (6 μm) and mounted onto

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Abbreviations: GnRH, gonadotropin-releasing hormone; GnRH-R, GnRH receptor; FSH, follicle-stimulating hormone; LH, luteinizing hormone; GH, growth hormone; PRL, prolactin; SL, somatolactin; DIG, digoxigenin; RT-Q-RT-PCR, real-time quantitative RT-PCR; LCM, laser-capture microdissection; TSH, thyroid-stimulating hormone; POMC, proopiomelanocortin; ACTH, adenocorticotropin; MSH, melanocyte-stimulating hormone.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AB111356 (GnRH-R1), AB111357 (GnRH-R2), and AB158490 (GnRH-R3)].

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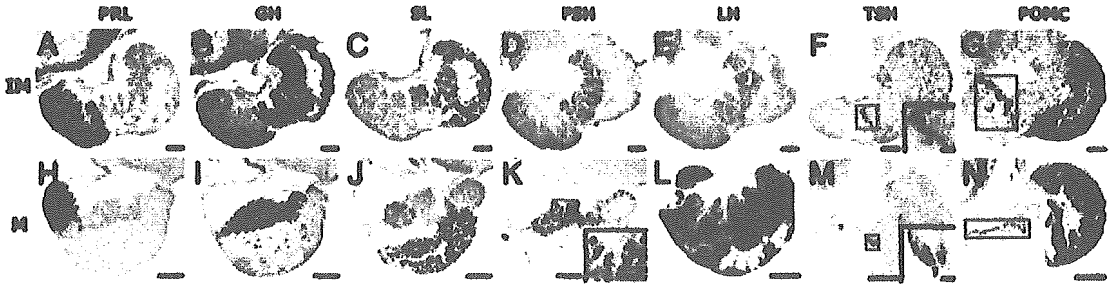

Fig. 1. Photomicrographs of DIG-labeled endocrine cells of the pituitary. Immature (IM) (*A–G*) and mature (M) (*H–N*) males are shown. (*A* and *H*) PRL cells. (*B* and *I*) GH cells. (*C* and *J*) SL cells. (*D* and *K*) FSH cells. (*E* and *L*) LH cells. (*F* and *M*) TSH cells. (*G* and *N*) POMC (MSH, ACTH); ACTH cells are shown in rectangles. (Scale bars: 70 μm in *A–G*; 200 μm in *H–N*; 20 μm in *F* and *M Insets*; and 70 μm in *K Inset*.) Cells shown in *F*, *K*, and *M Insets* are from within the rectangles.

aminopropyl triethoxy silane-treated slide glass (Matsunami Glass, Tokyo) and stored at −80°C until use.

For DIG *in situ* hybridization, we cloned and identified partial sequences of SL (GenBank accession no. AB120767; unpublished data) and thyroid-stimulating hormone β subunit (TSHβ, GenBank accession no. AB120769; unpublished data) whereas the sequences of other tilapia pituitary hormones were obtained from GenBank [accession nos.: M27010 (PRL_{188}), M97766 (GH1), AF289173 (*FSHβ subunit*), AY294016 (*LHβ subunit*), and AF116240 (proopiomelanocortin, POMC; adenocorticotropin, ACTH; and melanocyte-stimulating hormone, MSH)]. Because GH1 and GH2 encode an identical polypeptide (13) and PRL_{188} and PRL_{177} are colocalized in the same pituitary cells in the tilapia (14), we used the sequences of GH1 and PRL_{188} to synthesize riboprobes for *in situ* hybridization. Sense and antisense riboprobes were synthesized by using the pGEM-T easy transcription vector constructs (Promega), linearized with *Spe*I or *Nco*I endonuclease (Nippon Gene) as a template for T7 or SP6 RNA polymerase (Toyobo, Tokyo). The RNA probes were labeled by using DIG RNA-labeling mix (Roche Diagnostics). DIG *in situ* hybridization was carried out as described (4).

LCM of Pituitary Cells. The hydrated pituitary section was overlaid with a thermoplastic membrane mounted on an optically transparent cap (CapSure Macro LCM Caps, Arcturus, Mountain View, CA). We used a Pix Cell II Laser capture instruments (Arcturus), to microdissect DIG-identified pituitary cells by focal melting of the membrane through laser activation (laser pulse power, 25–65 mW; laser pulse duration, 1.5 ms; laser spot size, 10-μm diameter). Heat-pulled borosilicate glass microcapillary pipette (1.5-mm outer diameter, Harvard Apparatus, Edenbridge, Kent, U.K.; micropipet puller, Type PE-2, Narishige, Tokyo) attached to a micromanipulator (Narishige) was used to remove undesirable tissue around the periphery of the single cells. Then, by using a negative pressure, single-cells were harvested from the LCM cap into the micropipette under visual control and subsequently expelled into a sterile 1.5-ml reaction tube containing 50 μl of the lysis buffer and stored at −80°C until total RNA isolation. For unbiased cell sampling, 8–10 cells were harvested at random (≈2 cells per alternate section) along the rostral-caudal extent of the whole population of each pituitary cell type in each animal ($n = 5$ per age group). Only those cells positive for each pituitary hormone and free from genomic contamination were used for RT-Q-RT-PCR analysis ($n = 5$–9 cells per animal; 23–44 cells per pituitary cell type per age group).

GnRH-R Types in Pituitary Cells. The cell harvesting protocol and the conditions for RT-PCR were similar to those described elsewhere (12, 15). Briefly, the harvested single-cell from the pituitary was digested with 1 μg of proteinase K (Gentra Systems, Minneapolis, MN) and 10 units of ribonuclease inhibitor (Eppendorf, Hamburg, Germany) for 1 h at 53°C. The cell lysate was incubated for 1 h at 37°C with 1 unit of ribonuclease-free DNase I (Promega) to eliminate genomic DNA and heat denatured at 95°C for 10 min to separate the mRNA from the DIG-labeled riboprobe. Total RNA was extracted from the cell lysate by using ISOGEN (Nippon Gene) and reverse transcribed to cDNA with 0.1 pmol of random primers (TaKaRa) by using 40 units of SuperScript III reverse transcriptase (Invitrogen).

To confirm the presence of GnRH-Rs and pituitary hormone transcripts, the single cell's cDNA was subjected to RT-PCR using gene-specific primers for GnRH-Rs and pituitary hormones [GenBank accession nos.: AB120767 (SL), AB120769 (TSHβ), M27010 (PRL_{188}), M27011 (PRL_{177}), M97766 (GH1), M97765 (GH2), AF289173 (FSHβ), AY294016 (LHβ), and AF116240 (POMC); Table 2, which is published as supporting information on the PNAS web site] and 1/20th of a single cell's RT cDNA solution. To confirm the sequences, some bands were subcloned and both strands of the DNA were sequenced as described above. Several controls were included for the RT-PCR: buffer without harvested cells and no reverse transcriptase.

Quantitative Analysis of GnRH-Rs in Pituitary Cells. To quantify copies of GnRH-R transcripts in pituitary cells, cDNAs from single cells were subjected to RT-Q-RT-PCR, which was performed in 10-μl reaction volumes consisting of 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM GnRH-R primers, and 200 nM GnRH-R hybridization probes (GR7 through GR15; Table 2), and 1/20th of a single cell's RT cDNA or absolute standard cDNA by using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The PCR conditions were as described (12, 15). The GnRH-Rs hybridization probes spanned an intron and complemented the sequence on either side of the splice site of the gene. For each animal and pituitary cell type, average copies of transcripts per cell were determined, and these values were combined to give experimental group means. All values are expressed as the mean ± SEM, and statistical comparisons were made between immature and mature males ($n = 5$ per group) by using nonparametric ANOVA followed by Fisher's probable least-squares difference test. $P < 0.01$ or $P < 0.05$ were considered statistically significant.

Results

DIG *in Situ* Hybridization for Pituitary Hormones. In immature and mature males, DIG *in situ* hybridization for pituitary hormones (Fig. 1) showed cells expressing PRL_{188} mRNA localized in the rostral pars distalis (RPD, Fig. 1 *A* and *H*). GH cells were seen in the dorsal and LH cells in the ventral proximal pars distalis of mature males (PPD; Fig. 1 *B*, *I*, and *L*). The expression of LHβ transcripts was undetectable by *in situ* hybridization in immature males (Fig. 1*E*). FSH cells were scattered among GH cell popula-

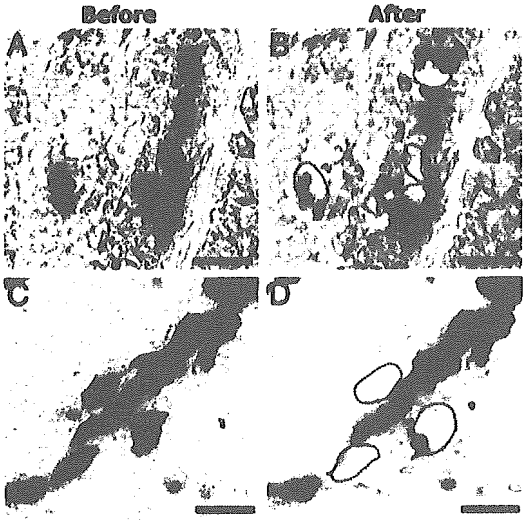

Fig. 2. DIG-labeled FSH (*A* and *B*) and GH (*C* and *D*) cells before and after LCM. (Scale bar, 20 μm.)

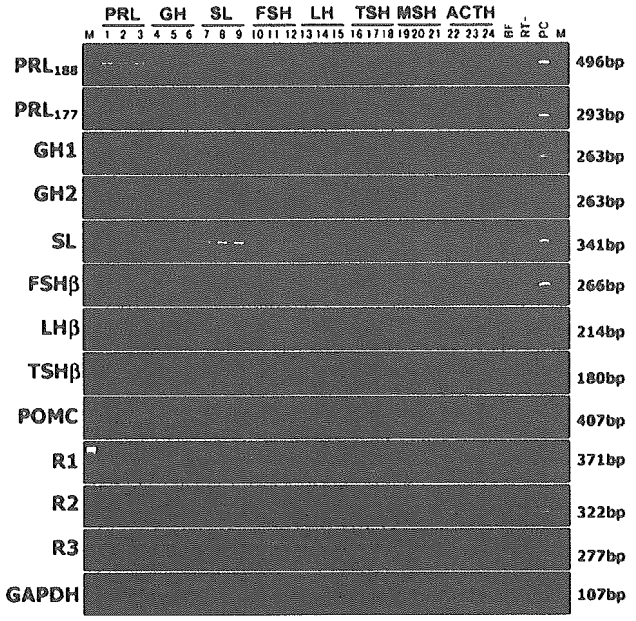

Fig. 3. Composite gel showing expression of amplicons of pituitary hormones and GnRH-Rs (R1, R2, and R3) in representative PRL cells (lanes 1–3), GH cells (lanes 4–6), SL cells (lanes 7–9), FSH cells (lanes 10–12), LH cells (lanes 13–15), TSH cells (lanes 16–18), MSH cells (lanes 19–21), and ACTH cells (lanes 22–24) taken from mature males. BF, buffer control; RT−, without reverse transcriptase; PC, whole pituitary cDNA as positive control for PCR; M, marker, DNA 100-bp size ladder. Note that the expressions of PRL_{188} and PRL_{177} and GH1 and GH2 transcripts are colocalized in PRL and GH cells, respectively. The POMC primer recognizes both MSH and ACTH transcripts. The sizes of the bands, in base pairs, are given in the right margin.

tion in the PPD (Fig. 1 *D* and *K*). TSH cells were located at the boundary between PPD and the RPD (Fig. 1 *F* and *M*). SL cells were located along the edge of the neurohypophysis in the pars intermedia (PI; Fig. 1 *C* and *J*). The POMC probe hybridized with both ACTH and MSH cells. ACTH cells were located in the dorsal periphery of the RPD in contact with the neurohypophysis and MSH cells were located in the PI (Fig. 1 *G* and *N*).

GnRH-R Types in Pituitary Cells. There was no genomic DNA contamination in the harvested single-cells of the pituitary (Figs. 2 and 3). RT-PCR showed that 70–85% of DIG-labeled pituitary cells had pituitary hormone transcripts (Fig. 3). The amplicon sizes of PRL_{188}, PRL_{177}, GH1, GH2, SL, FSHβ, LHβ, TSHβ, and POMC and their sequences were identical with tilapia pituitary hormones (Fig. 3; see *Materials and Methods* for GenBank accession numbers). Because PRL_{188} and PRL_{177} and GH1 and GH2 transcripts were colocalized (Fig. 3), these are described as single PRL and GH molecules throughout the text. Nested PCR was necessary to observe R1, R2, and R3 in pituitary cells. The amplicon sizes of R1 (371 bp), R2 (322 bp), and R3 (277 bp) and their sequences were identical with tilapia GnRH-Rs (see *Materials and Methods* for GenBank accession numbers). RT-PCR revealed that 40–60% of all pituitary cell types had GnRH-Rs (Fig. 3).

Quantitative Analysis of GnRH-Rs in Pituitary Cells. RT-Q-RT-PCR showed that 58–88% of pituitary cells in mature males expressed single or multiple GnRH-R transcripts (Table 1). In addition, a large variation in copies of R1, R2, and R3 transcripts were observed between individual cells (Fig. 4 *A–H Left*). R1 and R2 transcripts were predominant in the two reproductive states (Fig. 4 *A–H Right*). Absolute copies of GnRH-R transcripts in TSH and ACTH cells were below detectable levels in immature males (Fig. 4 *F* and *H*).

GH Family (PRL/SL/GH). ***PRL cells.*** The total percentage of PRL cells in immature (77%, 33 of 43 cells) and mature (84%, 24 of 28 cells) males expressing single or multiple GnRH-Rs were statistically nonsignificant between the two reproductive states (n = 5 per age group; Table 1).

The absolute copies of R1 (mature, 37,478.3 ± 9,965.8 vs. immature, 12,201.3 ± 3,299.9 copies per cell) and R2 transcripts (mature, 17,779.2 ± 4,436.2 vs. immature, 3,275.0 ± 882.0 copies per cell, $P < 0.05$) were significantly higher in PRL cells in mature males (Fig. 4*A*).

GH cells. The total percentage of GH cells in immature (64%, 21 of 33 cells) and mature males (58%, 19 of 33 cells) expressing single or multiple GnRH-Rs were statistically nonsignificant between the two reproductive states (n = 5 per age group; Table 1). The R2+R3 combination was absent in GH cells in mature males (Table 1).

Absolute copies of R1, R2 and R3 transcripts in GH cells were nonsignificant between the two reproductive states (Fig. 4*B*).

SL cells. The total percentage of SL cells in immature (62%, 21 of 34 cells) and mature males (88%, 20 of 23 cells) expressing single or multiple GnRH-Rs in the two reproductive states were statistically nonsignificant (n = 5 per age group; Table 1). A significantly higher percentage of SL cells in mature males had R1+R2 (42.0 ± 10.1% vs. 11.4 ± 11.4%; $P < 0.01$, n = 5 per age group) but lacked R2 subtype (Table 1).

Absolute copies of R1 (mature, 51,048.1 ± 13,240.3 vs. immature, 10,469.0 ± 2,148.7 copies per cell), R2 (mature, 34,210.6 ± 10,625.0 vs. immature, 4,739.8 ± 1,007.4 copies per cell) and R3 transcripts (mature, 3,657.5 ± 1,403.0 vs. immature, 524.1 ± 112.1 copies per cell, $P < 0.05$) were significantly higher in SL cells in mature males (Fig. 4*C*).

Glycoproteins (FSH/LH/TSH). ***FSH cells.*** The total percentage of FSH cells expressing single or multiple GnRH-Rs were statistically nonsignificant in immature (89%, 39 of 44 cells) and mature (72%, 24 of 33 cells; Table 1) males. A significantly higher percentage of FSH cells in mature males had R1 (38.1 ± 10.5% vs. 16.1 ± 8.4%; $P < 0.01$; n = 5 per age group). A significantly higher percentage of FSH cells in immature males had R1+R2 (24.7 ± 9.5% vs. 6.2 ± 3.8%, $P < 0.05$) and R1+R2+R3

Table 1. Percentage of pituitary cells with single or multiple GnRH-R transcripts

		PRL	GH	SL	FSH	LH	TSH	MSH	ACTH
R1	IM	20.8 ± 4.1	11.4 ± 7.0	20.5 ± 7.2	**16.1 ± 8.4****	20.0 ± 5.0	0.0	23.2 ± 6.2	0.0
	M	18.7 ± 6.4	23.8 ± 7.5	9.0 ± 5.6	**38.1 ± 10.5**	12.5 ± 4.0	46.0 ± 11.7	35.0 ± 4.7	31.9 ± 8.1
R2	IM	13.9 ± 4.5	9.1 ± 3.7	6.2 ± 3.8	2.2 ± 2.2	7.5 ± 5.0	0.0	2.9 ± 2.9	0.0
	M	6.7 ± 4.1	18.1 ± 7.4	0.0	15.7 ± 7.0	15.0 ± 4.7	0.0	2.5 ± 2.5	9.0 ± 5.9
R3	IM	2.2 ± 2.2	6.7 ± 6.7	8.6 ± 5.7	13.6 ± 5.4	12.5 ± 4.0	0.0	10.7 ± 5.3	0.0
	M	3.3 ± 3.3	6.7 ± 4.1	5.0 ± 5.0	9.0 ± 3.7	12.5 ± 0.0	3.3 ± 3.3	10.0 ± 4.7	11.4 ± 5.3
R1+R2	IM	11.9 ± 4.0	12.9 ± 6.2	**11.4 ± 11.4****	**24.7 ± 9.5***	12.5 ± 4.0	0.0	22.5 ± 6.1	0.0
	M	24.0 ± 8.4	2.9 ± 2.9	**42.0 ± 10.1**	**6.2 ± 3.8**	2.5 ± 2.5	0.0	12.5 ± 5.6	11.9 ± 8.4
R1+R3	IM	8.9 ± 4.2	8.6 ± 3.5	6.2 ± 3.8	2.5 ± 2.5	10.0 ± 2.5	0.0	10.0 ± 2.5	0.0
	M	3.3 ± 3.3	3.3 ± 3.3	5.0 ± 5.0	0.0	5.0 ± 5.0	19.3 ± 9.0	12.5 ± 5.6	0.0
R2+R3	IM	2.2 ± 2.2	6.2 ± 3.8	2.9 ± 2.9	2.2 ± 2.2	10.0 ± 7.3	0.0	2.5 ± 2.5	0.0
	M	3.3 ± 3.3	0.0	5.0 ± 5.0	0.0	15.0 ± 9.2	0.0	0.0	2.9 ± 2.9
R1+R2+R3	IM	16.9 ± 7.4	9.1 ± 3.7	6.2 ± 3.8	**25.3 ± 4.7****	7.5 ± 5.0	0.0	12.9 ± 4.0	0.0
	M	24.7 ± 12.8	3.3 ± 3.3	22.0 ± 10.2	**3.3 ± 3.3**	10.0 ± 2.5	19.3 ± 6.4	0.0	0.0
R-positive	IM	77.0 ± 6.5	63.8 ± 7.5	61.9 ± 5.4	88.9 ± 6.1	80.0 ± 3.1	0.0	84.6 ± 4.6	0.0
	M	84.0 ± 11.7	58.1 ± 11.9	88.0 ± 8.0	72.4 ± 6.3	72.5 ± 7.3	88.0 ± 8.0	72.5 ± 7.3	67.1 ± 7.7
R-negative	IM	23.0 ± 6.5	36.2 ± 7.5	38.1 ± 5.4	**11.1 ± 6.1***	20.0 ± 3.1	0.0	15.4 ± 4.6	0.0
	M	16.0 ± 11.7	41.9 ± 11.9	12.0 ± 8.0	**27.6 ± 6.3**	27.5 ± 7.3	12.0 ± 8.0	27.5 ± 7.3	32.9 ± 7.7

R-positive and R-negative represent the total percentage of cells with and without GnRH-Rs, respectively. Statistical analysis on an animal basis ($n = 5$ per age group) show significant differences in the percentage of SL and FSH cells expressing GnRH-Rs in immature and mature males (bolded values). *, $P < 0.05$; **, $P < 0.01$, Fisher's probable least-squares difference test.

transcripts (25.3 ± 4.7% vs. 3.3 ± 3.3%, $P < 0.01$; $n = 5$ per age group) (Table 1).

Absolute copies of R1 (mature, 8,561.5 ± 3,600.2 vs. immature, 3,476.5 ± 472.7 copies per cell) and R3 (mature, 2,965.8 ± 707.6 vs. immature, 1,411.8 ± 221.9 copies per cell, $P < 0.05$) were significantly higher in FSH cells in mature males (Fig. 4*D*).

LH cells. The total percentage of LH cells expressing single or multiple GnRH-Rs were statistically nonsignificant between immature (80%, 32 of 40 cells) and mature males (73%, 29 of 40 cells; Table 1).

Absolute copies of single and multiple transcripts of GnRH-Rs were statistically nonsignificant between the two reproductive states (Fig. 4*E*).

TSH cells. TSH cells were devoid of GnRH-R transcripts in immature (0%, 0 of 30 cells) but not in mature (89%, 23 of 26 cells) males (Table 1). R1 type was dominant in TSH cells in mature males (R1, 46.0 ± 11.7% vs. R3, 3.3 ± 3.3%). However, mature males lacked TSH cells with R2, R1+R2 and R2+R3 transcripts (Table 1).

Absolute copies of R1 (mature, 30,687.9 ± 10,408.4 copies per cell) were significantly higher than R2 (mature, 7,930.1 ± 3,663.5 copies per cell) and R3 (mature, 2,568.3 ± 850.8 copies per cell; $P < 0.05$) transcripts in TSH cells in mature males (Fig. 4*F*).

POMC Family (MSH/ACTH). ***MSH cells.*** The total percentage of MSH cells in immature (85%, 33 of 39 cells) and mature (73%, 29 of 40) males expressing single or multiple transcripts of GnRH-Rs were statistically nonsignificant (Table 1). MSH cells lacked R2+R3 and R1+R2+R3 combination in mature males (Table 1).

Immature males had significantly higher absolute copies of R1 transcripts (immature, 12,394.8 ± 3,152.6 vs. mature, 3,477.7 ± 663.4 copies per cell, $P < 0.01$) but lower copies of R3 transcripts (immature, 320.7 ± 62.3 vs. mature, 863.9 ± 259.1 copies per cell, $P < 0.05$) compared to mature males (Fig. 4*G*).

ACTH cells. ACTH cells were devoid of GnRH-R transcripts in immature (0%, 0 of 38 cells) but not in mature (67%, 23 of 34 cells) males (Table 1). ACTH cells in mature males lacked R1+R3 and R1+R2+R3 combinations (Table 1).

Absolute copies of R1 were significantly higher than R3 transcripts (mature, 6,961.3 ± 1,440.3 vs. immature, 177.5 ± 108.0 copies per cell, $P < 0.01$) in ACTH cells in mature males (Fig. 4*H*).

Discussion

Localization. Localization using DIG *in situ* hybridization showed pituitary cells in the immature and mature male tilapia segregated into three distinct zones: rostral pars distalis (PRL and ACTH cells), proximal pars distalis (GH, LH, FSH, and TSH cells), and pars intermedia (SL and MSH cells). This perfect degree of correspondence between the distributions of transcripts and proteins confirms previous studies in tilapia (4, 16).

We have cloned and obtained full-length sequences for three GnRH-Rs in the tilapia *O. niloticus*, designated here as tilapia R1, R2, and R3 (unpublished data). We used single-cell RT-PCR to show that, in addition to FSH and LH cells (4), multiple GnRH-Rs are also present in PRL-, GH-, SL-, TSH-, ACTH-, and MSH-producing cells. We are aware that false positives and false negatives may occur from failure of cell harvesting and/or RT-PCR procedures. Therefore, the control measures that we undertook included the use of negative and positive PCR controls (see *Materials and Methods*). No products were detected in the buffer without harvested cells or without reverse transcriptase, and there was no genomic DNA contamination in the harvested single cells. Furthermore, the amplicon sizes and their sequences were identical with tilapia GnRH-Rs. Together, these results demonstrate the specificity of this procedure. Thus, the amplicons are authentic, and we are confident that the present results provide well controlled evidence for the presence of three GnRH-R transcripts in native pituitary cells.

Cellular and Functional Heterogeneity of GnRH-Rs. The present study provides evidence that subpopulations of pituitary cells express single (42.9%), multiple (32.4%), or lack (24.7%) GnRH-R transcripts and that the frequency with which we detected GnRH-R transcripts varied significantly across the two male reproductive states.

GH Family. Members of the GH family (PRL/SL/GH) have been grouped together because of their structural similarities (17). Our demonstration of multiple GnRH-R transcripts in PRL/SL/GH cells and the presence of GnRH-R protein 1B in PRL cells and R-III in GH cells supports the role of GnRH in the regulation of the GH family (4, 8, 11). In addition, several lines of evidence suggest that GnRH is a secretagogue of PRL/SL/

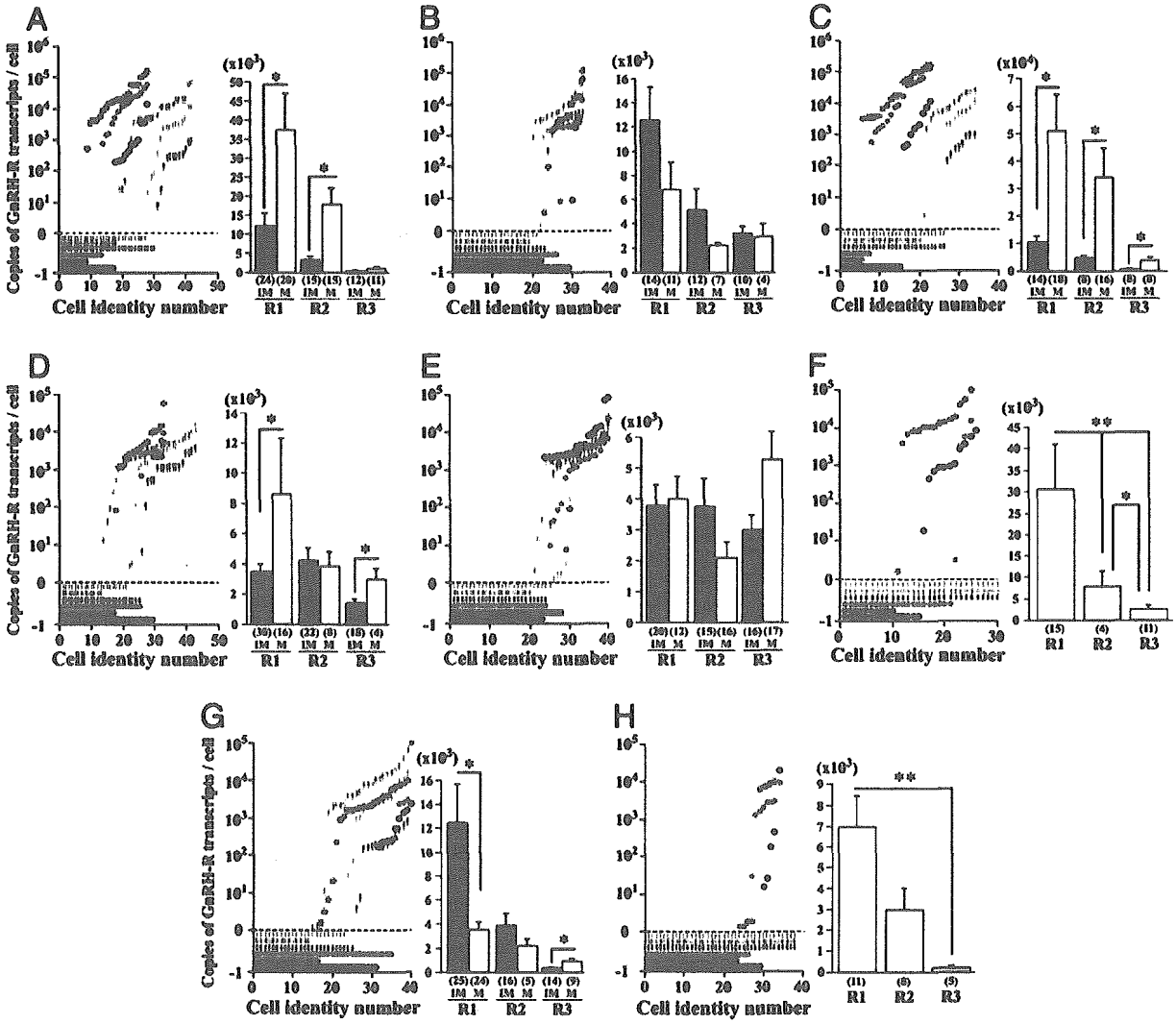

Fig. 4. Distribution of GnRH-R transcripts in individual pituitary cells. (*Left*) Graphs showing the distribution of R1 (red), R2 (green), and R3 (black) transcripts in individual cells expressing PRL (immature, 28 cells; mature, 43 cells) (*A*), GH (immature 33 cells; mature, 33 cells) (*B*), SL (immature 34 cells; mature, 23 cells) (*C*), FSH (immature, 44 cells; mature, 33 cells) (*D*), LH (immature, 40 cells; mature, 40 cells) (*E*), TSH (immature, 30 cells; mature, 26 cells) (*F*), MSH (immature, 39 cells; mature, 40 cells) (*G*), and ACTH (immature, 38 cells; mature, 34 cells) (*H*) taken from immature (short bars) and mature (circles) males. The *x* axis represents cell identity numbers and the *y* axis represents copies of GnRH-R transcripts per cell. The short bars and circles below zero are undetectable levels of GnRH-Rs in positively identified pituitary cells. (*Right*) Histograms showing the average copies of R1, R2, and R3 transcripts per cell deduced from the total number of cells expressing PRL (*A*), GH (*B*), SL (*C*), FSH (*D*), LH (*E*), TSH (*F*), MSH (*G*), and ACTH (*H*) taken from immature (IM, filled bars) and mature males (M, open bars). Statistical comparisons are per cell basis using nonparametric ANOVA followed by Fisher's probable least-squares difference test. *, $P < 0.05$; **, $P < 0.01$. Cell numbers are given in parentheses.

GH. First, GnRH binding sites have been detected in PRL/SL/GH cells in teleosts (18, 19). Furthermore, GnRH stimulates PRL (6), SL (7), and GH release (8, 20) in teleosts.

In teleosts, PRL is primarily an osmoregulatory hormone (21) and SL is an environmental adaptation hormone (22). However, there is substantial evidence supporting the role of PRL and SL in steroidogenesis and gonadal maturation (23–25). Furthermore, PRL-binding sites and PRL receptors have been detected in the testis of tilapia *Oreochromis mossambicus* (26). Here, the significant increase in copies of R1 and R2 transcripts in PRL and SL cells in mature males may be involved in the mechanism of activation of PRL and SL release during gonadal maturation, which is supported by activation of PRL and SL genes (25). The absence of any difference in GnRH-R transcripts in GH cells between the two reproductive states suggests a role for GH during early and late testicular development. In contrast, in females, greater levels of GH receptor mRNA have been reported in immature oocytes in the tilapia (27) and in salmonids (28, 29). Whether these differences between males and females are also paralleled by sexually dimorphic GH secretion during development, as in mammals (30), which could initiate the difference in the timing of sex differentiation between tilapia females (28 days) and males (50 days) (see ref. 16) remains to be investigated.

Glycoprotein Family. Members of the glycoprotein family include FSH, LH, and TSH. To be active, FSHβ, LHβ, and TSHβ need to heterodimerize with the α-glycoprotein subunit (31). GnRH-R proteins (IA and IB) have been reported only in LH cells because of the difficulty to distinguish them from FSH cells immunocytochemically (due to lack of specific antibodies) (4).

The present study demonstrates multiple GnRH-Rs distinctly in FSH and LH cells, which suggests that the synthesis and release of FSH and LH hormones can be regulated by multiple GnRH ligands. The expression of multiple GnRH-Rs in TSH cells has not been described previously.

The percentage of FSH cells with R1+R2 and R1+R2+R3 combination was significantly higher in immature males, a reproductive stage during which FSH hormone predominates (32). These results support the hypothesis that, in immature males, multiple GnRH-R combinations modulate FSH for spermatogenesis and gonadal steroidogenesis. Furthermore, in mature males, significantly high percentage of FSH cells with R1 and greater GnRH-R transcripts (R1, R3) suggest that FSH hormone levels remain elevated in mature males to maintain spermatogenesis (33).

The percentage of LH cells with GnRH-Rs and the copies of GnRH-R transcripts in LH cells were similar in the two reproductive states. This finding suggests that both LH and FSH hormones could have physiological roles in spermatogenesis and spermiation in male tilapia (34), in contrast to salmonids, in which FSH hormone is important for early gametogenesis and LH is important only for gonadal maturation (32, 33).

Although there is no evidence for GnRH in the regulation of TSH cells, the localization of thyroid hormone receptors in GnRH neurons (35) and the regulation of GnRH gene expression by thyroid hormones (36) demonstrate a relationship between GnRH and TSH cells. In immature males, the differentiation of TSH cells in the absence of GnRH-Rs suggests that GnRH might be important for release rather than synthesis of TSH hormone. On the other hand, in mature males, the activation of GnRH-Rs in TSH cells could have some role in gonadal maturation because thyroid hormones and TSH cells have been implicated in reproductive processes (37).

POMC Family. Our demonstration of multiple GnRH-R transcripts in ACTH and MSH cells suggests the role of GnRH in the regulation of the POMC-derived family of peptides from ACTH cells (16K fragment, ACTH, γ-lipotropin and β-endorphin) and MSH cells (α-MSH, β-MSH, γ-lipotropin, β-lipotropin and β-endorphin) (38, 39). There are leads in the literature that suggest GnRH1 stimulates ACTH and β-endorphin (40). Therefore, the significantly high R1, R2, and R3 transcripts in ACTH cells in mature males suggests their role during testicular maturation, which may include territoriality and aggressiveness because ACTH induced cortisol has been implicated in social dominance in cichlid fish (41). The role of α-MSH in steroidogenesis and timing of puberty and β-endorphin in reproductive behavior in rats are well documented (42, 43). Therefore, the greater copies of R1 and R3 transcripts in MSH cells in immature and mature males, respectively, suggests differential use of GnRH-Rs, probably to regulate different products of the POMC family of peptides during early and late stages of testicular development.

Summary. Given that pituitary cells are clustered, the present single-cell approach is technically more reliable than *in situ* hybridization to observe changes in absolute copies of transcripts in individual cells because it avoids the problem of overlap of silver grains from neighboring cells and demonstrates the presence of "silent pituitary cells" with undetectable levels of GnRH-R transcripts during the two reproductive states. Furthermore, this approach allows correlation of multiple gene expression profiles, which would greatly facilitate our understanding of the complex interactions that exist within individual pituitary cells. The presence of GnRH-Rs in MSH, ACTH, and TSH cells are noteworthy, and suggests their role in reproduction and/or novel roles for GnRH molecules in nonreproductive functions during gonadal development.

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