Long-term dosing studies using mutagenic carcinogens indicate a highly significant correlation between elevations in the level of rat glutathione *S*-transferase P messenger RNA and liver tumours of hepatocellular origin

S. E. Hilary RUSSELL,* Colin PEARSON,† Michael KELLY,† Shirley McQUAID* and Peter HUMPHRIES*‡

*Department of Genetics, Lincoln Place Gate, Trinity College, Dublin 2, Ireland, and †Central Toxicology Laboratory, Imperial Chemical Industries PLC, Alderley Park, Macclesfield, Cheshire SK10 4TJ, U.K.

We have investigated levels of transcript homologous with glutathione *S*-transferase P (GST-P; GST 7-7) in tumours and hyperplastic lesions induced in the livers of rats by long-term gavage dosing with diethylnitrosamine (DEN) and 6-*p*-dimethylaminophenylazobenzothiazole (6BT). Detailed histopathological examination of the livers of the 90 animals used in this study at 6–8 months after initiation of daily dosing revealed that, of the 30 animals treated with carcinogen, 15 had developed tumours or hyperplastic lesions. Of these, 11 were areas of fibrosarcoma/fibrous hyperplasia. The remaining four were hepatocellular carcinomas. Northern blotting of total RNA purified from these tissues revealed the presence of transcripts of 3 and 0.75 kb. Evidence is presented to indicate that the former is a hitherto-undetected precursor of the 3-kbp rat GST-P gene, the latter representing the previously characterized mature GST-P transcript. Large elevations of the 0.75-kb transcript (30–35-fold) were encountered in all of the hepatocellular carcinomas, but in none of the other lesions, indicating a highly significant correlation ($P < 0.001$) between high elevations in levels of GST-P mRNA and liver tumours of hepatocellular origin. Minor elevations in transcript level (≤ 5 -fold) were encountered in several of the non-hepatocellular lesions. In regenerating livers, small increases in the level of the 3-kb transcript (~ 3 -fold) were routinely detected in total RNA from all partial hepatectomies, a concomitant decrease of approximately similar magnitude occurring in the 0.75-kb transcript, suggesting that minor elevations in levels of GST-P transcript, where encountered in non-hepatocellular lesions, are related to pre-neoplasia rather than to the proliferative rate of hyperplastic cells *per se*. The data extend previous observations, carried out largely using short-term regimes, to an analysis of transcripts homologous with GST-P in hyperplastic, pre-neoplastic and neoplastic lesions induced by long-term dosing with genotoxic carcinogens, and strongly lend support to the concept that high (30-fold) elevations in GST-P transcript correlate most strikingly with tumours of hepatocellular origin.

INTRODUCTION

The glutathione *S*-transferases (GSTs) are a group of related multifunctional proteins assuming a major role in the detoxification of carcinogenic agents by catalysing the alkylation of glutathione, rather than DNA, by carcinogens. Three major families, 'Ya', 'Yb' and 'Yf', may be distinguished on the basis of subunit composition and cross-reactivity, and approx. 30 forms of GST have been identified in rodents and in man, attributable to the interaction of subunits to form homo- and hetero-dimers (Jakoby, 1978; Kalinyak & Taylor, 1982; Pearson *et al.*, 1983).

One form of the enzyme, GST-P (GST 7-7), a homodimer of Yf subunits and previously identified as protein p26-6.9 (Sato *et al.*, 1984), has been suggested as a marker enzyme for pre-neoplastic cells in rat liver during chemical carcinogenesis (Kitahara *et al.*, 1984; Satoh *et al.*, 1985; Suguoka *et al.*, 1985). Moreover, GST- π , sharing a common antigenicity with rat GST-P, has been shown to be significantly increased in primary

human hepatomas and in secondary hepatic tumours derived from the stomach and colon (Soma *et al.*, 1986).

In model studies of rodent hepatocarcinogenesis, pre-neoplastic nodules and tumours have most frequently been induced by the technique of Solt & Farber (1976), in which a chemical carcinogen, a selective growth inhibitor and a generalized growth stimulus (e.g. partial hepatectomy) are sequentially employed. Under these conditions, pre-neoplastic nodules usually appear in the liver within 3–4 weeks, and levels of GST-P have been shown to be elevated in such lesions, irrespective of the type of carcinogen employed (Suguoka *et al.*, 1985). Tumour induction by gavage dosing is, by contrast, a much slower process, and usually results in the development of several tumour types in addition to pre-neoplastic hyperplasias, and it is upon the latter method that long-term animal studies on potential carcinogens are largely based (Elliot *et al.*, 1983). In view of these considerations, we have studied levels of GST-P transcript in tumours and hyperplasias induced in the livers of rats by dosing

Abbreviations used: GST, glutathione *S*-transferase; DEN, diethylnitrosamine; 6BT, 6-*p*-dimethylaminophenylazobenzothiazole; 1 $\times$ SSC, 0.15 M-NaCl/0.015 M-sodium citrate; (k)b(p), (kilo)base(-pair).

‡ To whom correspondence and reprint requests should be sent.

Fig. 1. Histopathological examination of carcinogenized rat livers

(a) Normal liver; (b) hepatocellular carcinoma; (c) haemangiosarcoma; (d) fibrous hyperplasia; (e) fibrous hyperplasia including oval-cell proliferation.

with diethylnitrosamine (DEN) and 6-*p*-dimethylamino-phenylazobenzothiazole (6BT) over periods of 6–8 months. Transcripts were found to be highly elevated in hepatocellular carcinomas and slightly elevated in several zones of hyperplasia/sarcoma. Levels of the mature GST-P transcript were generally decreased in partially hepatectomized livers, suggesting that where elevations are found in hyperplastic tissue, these relate to pre-neoplasia rather than to the proliferative rate of hyperplastic cells.

MATERIALS AND METHODS

Induction of rodent hepatocellular carcinomas

Hepatic tumours were induced in Alderley Park rats by gavage dosing with 6BT and DEN (7.5 mg/kg body wt. and 550 μ g per animal respectively) according to previously described procedures (Elliot *et al.*, 1983; Elcombe *et al.*, 1985).

A total of 90 animals were used in the study. Ten animals received 6BT, and two groups of ten control animals received either water or corn oil only. A group of 20 animals received DEN, and two groups of 20 additional control animals received either water or corn oil. The animals were killed at 6–8 months after initial dosing for histopathological examination of livers and extraction of RNA.

Partial hepatectomies

Partial (70%) hepatectomies were performed on Alderley Park rats as described previously (Higgins & Anderson, 1931). Animals, together with sham-operated controls, were killed at 1, 2, 3, 4, 24 and 48 h during the regenerative phase.

Extraction of RNA and Northern-blot analysis

RNA was extracted from rodent liver by the guanidinium/hot-phenol method described by Maniatis *et al.*

(1982). Samples of total RNA (10 μ g) were fractionated on 1.5%-(w/v)-agarose gels containing 2 M-formaldehyde as described by Barrett & Mahy (1984) and blotted directly on to Hybond N membranes (Amersham International). Pre-hybridization and hybridization conditions with nick-translated DNA were as described by the manufacturers. All Northern blots were hybridized in 50% (v/v) formamide at 42 °C overnight and washed to $0.1 \times$ SSC at 42 °C. Nick-translation was carried out in the presence of [32 P]dCTP by the method of Rigby *et al.* (1977) to provide DNA probes with specific activities ranging from 8×10^7 to 2×10^8 c.p.m./ μ g. Elevations in levels of the GST-P transcript were determined by densitometric scanning of autoradiograms. In addition, in order to ensure equality of transfer of RNA, Northern blots were also probed with an 18S ribosomal DNA probe (a plasmid containing a 1.9-kb insert derived from the mouse 18S ribosomal RNA gene) kindly provided by Dr. N. Arnheim, State University of New York, New York, NY, U.S.A.

Nuclear and polysomal fractions were prepared by the method of Wilkes *et al.* (1979). Nuclear and polysomal RNA was then extracted by the guanidinium/hot-phenol method (Maniatis *et al.*, 1982).

RESULTS AND DISCUSSION

Histological examination of the livers of the 30 carcinogen-treated rats removed at 6–8 months after initiation of 6BT or DEN dosing revealed the presence of neoplastic or hyperplastic lesions in 15 animals. Of these, four were hepatocellular carcinomas, three were sarcomas, of which one was an haemangiosarcoma, and the remainder were areas of fibrous hyperplasia (Fig. 1). No histopathological lesions were found in the livers of control animals.

Total RNA purified from such tissues was subjected to Northern-blot analysis using an insert from the recombinant DNA probe pGP5 (Suguoka *et al.*, 1985) carrying a cDNA copy of the rat GST-P gene. A representative autoradiograph is depicted in Fig. 2. As described by Suguoka *et al.* (1985), the principal GST-P transcript detected was of 750 nucleotides. An additional transcript of 3 kb was also detected in many RNA species, particularly on longer autoradiographic exposure (see below). The level of GST-P mRNA in normal liver was low, but readily detectable (Fig. 2, lanes 1 and 4). In all of the hepatocellular carcinomas, two of which are illustrated in Fig. 2 (lanes 5 and 6), there was an elevation of the 750-nucleotide (0.75 kb) transcript of approx. 35-fold. Minor elevations in level of GST-P transcript were also found in several zones of fibrous hyperplasia/fibrosarcoma (e.g. Fig. 2, lane 2). In order to examine the possibility that moderate elevations such as these may be related directly to the proliferative rate of hyperplastic cells, we examined levels of GST-P transcript in rapidly regenerating liver tissue. Total RNA from rat livers at various times after 70% partial hepatectomy was electrophoresed and examined by Northern-blot analysis with the GST-P insert from plasmid pGP5. Control animals undergoing sham operations at each time point were also examined in order to exclude the possibility that surgical stress alone, without partial hepatectomy, may induce variations in the level of GST-P transcripts. The probe revealed transcripts of 0.75 and 3 kb. The results from the 4-h time point representative also of the

Fig. 2. Northern blot of total RNA extracted from rat livers, hybridized to GST-P probe

Lanes 1–6, RNA from normal liver, haemangiosarcoma, normal liver, normal liver, hepatocellular carcinoma and hepatocellular carcinoma respectively. Amounts of RNA loaded, specific radioactivity of the probe, conditions of hybridization and stringency of washing are provided in the Materials and methods section.

Fig. 3. Northern blot of total RNA extracted from regenerating rat livers (a) and of polysomal and nuclear RNA (b) hybridized to GST-P probe

(a) Total RNA extracted from sham-operated and partially hepatectomized animals at 4 h after partial hepatectomy (lanes 1 and 2 respectively) was hybridized with GST-P probe. (b) Total RNA was prepared from part of one rat liver (lane 3). The remainder of the liver was fractionated into polysomes and nuclei from which RNA was prepared (lanes 1 and 2 respectively).

1, 2, 3, 24 and 48 h time points, are illustrated in Fig. 3(a). In order to illuminate the 3-kb transcript in this, and in Fig. 3(b), longer autoradiographic exposures were required, producing a somewhat darker background. Nevertheless, both the 3-kb and 0.75 kb transcripts are clearly visible. No increase in the amount of the 750-nucleotide transcript occurred in regenerating liver. Rather, a decrease of up to 3-fold as compared with sham-operated controls was observed (e.g. Fig. 3a, lane 2). The 3-kb transcript may be a precursor of GST-P mRNA or the product of an unrelated gene bearing structural homology with GST-P. We favour the former hypothesis, in view of the fact, firstly, that, in genomic DNA, the rat GST-P gene is 3 kb in length (Okuda *et al.*, 1987). Secondly, when nuclear and polysomal RNA fractions were examined for transcripts homologous with GST-P, the nuclear fraction was found to be enriched for the 3 kb transcript, whereas the polysomal fraction was enriched for the 0.75 kb transcript (Fig. 3b). In addition, total RNA probed with DNA from the recombinant plasmid pSS0.6, carrying a 600-bp segment of rat genomic DNA from intron 5 of the GST-P gene (kindly provided by Dr. M. Sakai and Dr. M. Muramatsu; Okuda *et al.*, 1987) illuminated a transcript of approx. 3 kb (results not shown). In all regenerating liver samples, as compared with control animals undergoing sham operations at similar time points, an elevation in the level of the 3-kb transcript (up to 3-fold) was observed (e.g. Fig. 3a, lane 2). The reason for the build-up in the 3-kb transcript in regenerating tissue and the concomitant decrease in mature transcript is at present unknown.

In conclusion, our data indicate that moderate

Fig. 4. Hybridization of total RNA from human bladder carcinomas and rat liver to rat GST-P probe

Total RNA was extracted from two human bladder carcinomas (lanes 1 and 2) and from rat liver (lane 3). RNA species were subjected to Northern-blot analysis as described in the Materials and methods section and probed with rat GST-P cDNA insert.

elevations (≤ 5 -fold) in levels of GST-P transcript, where they occur in hyperplasias and/or fibrosarcomas, are related to pre-neoplasia rather than to the proliferative rate of hyperplastic cells and are in support of the concept that high elevations in GST-P transcripts (i.e. ≥ 30 -fold) are a characteristic feature of hepatocellular carcinomas whether induced by rapid techniques or, as in the present study, by long-term gavage dosing.

Finally, it is noteworthy that sufficient homology exists between the rat and human GST-P genes to permit the detection of a homologous transcript in total RNA from human bladder carcinomas (Fig. 4). An homologous transcript is also readily detectable in total RNA from peripheral white cells, bone marrow and prostate tissue, and in all cases is the same size as the rat 0.75 kb transcript (not shown). Since levels of GST-P (GST- π) protein have been shown to be elevated in some human tumours (Soma *et al.*, 1986), these observations suggest that the rat probe could readily be used to assess levels of transcript in RNA from malignant human sources.

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