Expression of the Immediate-Early Genes, c-fos, c-jun, and c-myc: A Comparison in Rats of Nongenotoxic Hepatocarcinogens with Noncarcinogenic Liver Mitogens

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The involvement of the immediate-early (IE) genes c-fos, c-jun, and c-myc in regenerative liver hyperplasia is accepted, but their involvement in direct hyperplasia is uncertain. We have examined the hypothesis that the ability to induce IE genes may reflect the hepatocarcinogenic potential of a chemical. The ability of 1,4-dichlorobenzene (DCB) (300 mg/kg) (a noncarcinogenic rat liver mitogen), diethylhexyl phthalate (DEHP) (950 mg/kg), and chloroacetic acid (120 mg/kg) (both nongenotoxic hepatocarcinogens) to induce c-fos, c-jun, and c-myc expression in rat liver was determined by Northern blot analysis and by in situ hybridization. Results were correlated to hepatic labeling index (LI) as determined by incorporation of BrdU in each of three lobes for each of three male F344 rats per group. Carbon tetrachloride (CCL4) (2 ml/kg) was used as a positive control. Increased LI was preceded by elevated expression of all three IE genes after CCL4, but also after DCB and DEHP, although induction by these was less marked. In all cases, there was considerable interanimal variation within groups, but little interlobe variation. Interestingly, there was a good correlation (r² = 0.85) between c-myc expression and LI, but not between LI and c-fos or c-jun. Despite the disparate carcinogenic potential of DEHP and DCB, both chemicals induced similar patterns of IE gene expression, suggesting that this cannot distinguish hepatocarcinogenic liver mitogens from nongenocarcinogenic liver mitogens. These data assist in the evaluation of IE gene expression both as a marker of direct versus regenerative hyperplasia and as an indicator of the hepatocarcinogenic potential of liver mitogens. © 1997 Society of Toxicology.

Nongenotoxic carcinogens cause tumors without damaging DNA. Although their mechanisms of action remain to be determined, there is a good correlation between the ability of a nongenotoxic chemical to induce replicative DNA synthesis in the liver and its subsequent carcinogenicity (Ashby et al., 1994; Cunningham, 1996). For example, the antihista-

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hepatocarcinogenic DNA synthesis. Previously, it has been suggested that the pattern of expression of immediate–early (IE) gene expression may differ depending on the nature of the proliferative stimulus (Coni et al., 1990; Goldsworthy et al., 1994). During regenerative liver growth induced by partial hepatectomy or carbon tetrachloride (CCl₄), the increase in labeling index (LI) is clearly preceded by increased expression of these genes. However, direct liver growth induced by some mitogens appears to proceed in the absence of a detectable increase in the expression of c-jun, c-fos, and c-myc (Coni et al., 1990; reviewed by Columbano and Shinozuka, 1996). Recent data have suggested that such apparent differences might, in part, be attributed to interanimal variation and/or to the extent of the proliferative response (Holden et al., 1997a). To investigate further IE gene expression preceding low versus high risk S-phase, we have investigated the effect of three chemicals, each with different profiles of LI and carcinogenicity, on the expression of c-jun, c-fos, and c-myc.

The PP, DEHP, a plasticiser used in PVC manufacture, and chlorendic acid (CEA), a flame retardant and intermediate in the manufacture of polyester resins, are unequivocally hepatocarcinogenic in Fisher 344 rats in National Toxicology Program cancer studies (NTP 1982, 1987a). In contrast, DCB, a widely used deodorizer and disinfectant, was not hepatocarcinogenic in rats (NTP 1987b). Previous studies in this laboratory have shown that, at the dose regimes used in the NTP studies, DEHP induced an initial wave of DNA synthesis during the first 7 days of dosing, while CEA induced chronic S-phase from 28 days which was sustained for at least 365 days of dosing (Odum et al., 1997). Surprisingly, DCB induced a profile of hepatocyte DNA synthesis almost identical to that of DEHP (Odum et al., 1997) despite differences in their carcinogenicity in the rat liver at NTP dose levels. We have used Northern blot analysis to compare the effects of these three test chemicals on IE gene expression in the liver. CCl₄, which causes regenerative hyperplasia was used as a positive control since CCl₄ treatment gives significant induction of c-jun, c-fos, and c-myc (Herbst et al., 1991; Coni et al., 1993; Goldsworthy et al., 1994). In addition, we have correlated expression detected by Northern blotting to cellular localisation by in situ hybridization. Three lobes (caudate, left lateral, and right median) were examined in each of three animals per group. The data assist in our understanding of IE gene expression prior to hepatic S-phase induced by nongenotoxic carcinogens.

METHODS

Chemicals. DCB, DEHP, and CEA were obtained from Aldrich Chemical Company. Chlorendic acid was purchased as the anhydride and hydrolyzed within Zeneca CTL. Unless stated otherwise, all other chemicals were obtained from Sigma and were of the highest available purity.

Animals and animal procedures. Male Fischer 344 rats (6–8 weeks old on arrival) were purchased from Harlan Olac Ltd. and acclimatised to appropriate control diet for 1 week prior to dosing. Animals were housed three per cage, in appropriate conditions of temperature and humidity and with a 12-h light/dark cycle. The study consisted of an untreated group, a vehicle (corn oil) control, positive control (CCl₄), and three treatment groups. Dose levels were chosen to correlate with those used in NTP bioassays (NTP, 1982, 1987a,b) and in previous studies in this laboratory (Odum et al., 1997). DEHP and CEA were previously administered in the diet, a route not compatible with determination of IE gene expression 1 h after dosing. Therefore, gavage doses equivalent to the average daily doses calculated from food consumption data were used. DEHP (950 mg/kg), CEA (120 mg/kg), DCB (300 mg/kg), and CCl₄ (2 ml/kg) were administered as a single gavage dose in corn oil. Food and water were available ad libitum.

Hepatic LI. Bromodeoxyuridine (BrdU) (15 mg/ml in saline) was administered by continuous infusion from Alzet (Alza, Palo Alto, Ca.) osmotic minipumps implanted subcutaneously 48 h prior to dosing. Hepatic LI was determined on three animals per group 48 h after dosing. Sections of caudate, right median, and left lateral lobes from each animal were fixed for 24 h in formalin prior to processing and detection of LI by the method of Soames et al. (1994).

Experimental design. IE gene expression was determined on three animals per group terminated 1 h postdosing by exposure to a rising concentration of CO₂, followed by exsanguination. Parallel sections of caudate, right median, and left lateral liver lobes were fixed in 4% paraformaldehyde for 24 h and processed for in situ hybridization (ISH). Parallel sections of liver (4–600 mg) were flash frozen for RNA extraction and Northern blot analysis.

Northern blot analysis. Total RNA extracted with total RNA isolation reagent (Advanced Biotechnologies Ltd) was run on 1.2% agarose/0.22 M formaldehyde/Mops gels and capillary blotted overnight onto Hybond N⁺ nylon membrane (Amersham, UK). Filters were UV cross-linked and prehybridized in RapidHyb (Amersham) (1 h at 65°C) prior to hybridization (3–4 h at 65°C). cDNA probes for c-fos, c-jun, and c-myc (kindly supplied by P. Holden, (Holden et al., 1997a,b)) were 32P labeled using HighPrime (Boehringer Mannheim, Lewes, UK) and purified prior to use (Nucleotide Removal Kit, Qiagen). Filters were washed in 0.5 × SSC/0.1% SDS before exposure to autoradiographic film (Hyperfilm MP, Amersham). Expression of c-jun, c-fos, and c-myc were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression following densitometric analysis. Group means were compared to the mean for the corn oil control group using the Student t test. All statistical significances are expressed at the level of p ≤ 0.05.

In situ Hybridization. Sections were treated with proteinase K (Boehringer Mannheim, Lewes, UK) 15 μg/ml for 30 min at 37°C and postfixed in 0.4% paraformaldehyde (4°C for 20 min). The sections were prehybridized (1 h at 37°C) in a humid incubation chamber prior to hybridization with 500 ng/ml fluorescein labeled rat specific oligonucleotide probe (c-fos, R&D Systems, Abingdon, UK; c-myc and c-jun, Hybriprobe, TCS Biologicals, Botolph Claydon, UK). Sections pretreated with RNAase (1 mg/ml for 1 h at 37°C) or the omission of probe were used as negative controls. The sections were incubated overnight at 40°C in a humid chamber. The bound probe was detected using an anti-fluorescein detection system (Dako Hybridization Detection Kit, DAKO A/S, Denmark) following the manufacturer’s instructions. The number of labeled cells and lobular distribution was determined by light microscopy. The number of labeled cells in each lobe was scored using a semi-quantitative system (none, minimal, slight, moderate, or marked).

RESULTS

DNA Synthesis

Figure 1. Shows the profile of DNA synthesis induced during the 48h period following a single dose of chemical.
The positive control (CCl₄) induced significant S-phase (10- to 20-fold of corn oil control (CO)), and the treatments DCB and DEHP also induced S-phase (1.5- to 2.5- and 4- to 5-fold CO, respectively). However, CEA did not induce S-phase compared to CO controls. Distribution of LI was similar in all three of the liver lobes examined. There was considerable variation between animals within the same treatment group.

*Northern Blot Analysis of Expression of IE Genes*

Figures 2 and 3 show Northern blot analysis of the caudate and right median lobe, respectively, following hybridization with probes for c-jun, c-fos, c-myc, and GAPDH. Figure 4 shows mean gene expression quantified by image analysis for individual lobes. Figures 5–7 show gene expression for each animal in all three of the lobes examined. For all genes, there was considerable interanimal variation (up to twofold) within treatment groups; this variation was as large as that seen between treatment groups.

Expression of c-fos (Figs. 4a and 5) was increased in all lobes of all animals after CCl₄. Expression of c-fos was increased in some lobes of some animals treated with either DEHP or DCB. However, some animals in these groups did not respond (Fig. 5). DEHP treatment increased c-fos expression in the left lateral lobe in two of three animals (Fig. 5) in the group (~2-fold CO). DCB induced c-fos expression in the left lateral lobe of all rats (~2-fold CO) and right median lobe of two of three rats (~6- to 10-fold of CO).

Expression of c-jun (Figs. 4b and 6) was increased strongly in all lobes of all animals after CCl₄. Expression of c-jun was increased in all animals treated with either DEHP or DCB. However, c-jun expression was not increased in all of the lobes examined. Both DEHP and DCB increased slightly the expression of c-jun in the right median lobe of all animals (~2-fold and ~3-fold of CO values, respectively). DEHP and DCB also increased expression of c-jun in the left lateral (~2-fold of CO) and caudate lobe of one or two

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**FIG. 1.** Hepatocyte labeling index (LI) for three individual lobes of the liver as detected by bromodeoxyuridine incorporation. Values are mean ± SD, n = 3.

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**FIG. 2.** Northern blots of immediate–early gene expression in the caudate lobe of individual animals.

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**FIG. 3.** Northern blots of immediate–early gene expression in the right median lobe of individual animals.
Expression of *c-myc* was increased in all lobes of all animals after CCl₄, although the induction by CCl₄ was not as robust as the induction of *c-fos* and *c-jun* by CCl₄. The mean expression of *c-myc* (Figs. 4c and 7) in the right median lobe was increased (~2-fold of corn oil control values) in some animals treated with either DEHP or DCB. In contrast expression of *c-myc* in the left lateral and caudate lobes was higher, and the mean was comparable with that seen in the positive control (CCl₄) animals. In addition, *c-myc* expression was increased in all three lobes (>2-fold of CO) of a single animal treated with CEA (Fig. 7). Again, the variation in *c-myc* expression within treatment groups was as large as the variation between treatment groups, with the exception of CCl₄.

**Correlation of Gene Expression with LI**

Figure 8 shows expression of *c-myc* measured 1 h after dosing, plotted against LI for the 48-h time period after dosing. The correlation coefficient is shown with and without the positive control value. The expression of *c-myc* in any of the three animals within each treatment group (Fig. 6). There was a large range of expression within the caudate lobe of the control group (2-fold variation).

![Graphs of gene expression](image)
IMMEDIATE-EARLY GENE EXPRESSION

Expression of c-myc in control or corn oil animals was detected in the occasional cell, mainly in the midzonal region (Fig. 9b). As seen by Northern blot analysis, the number of cells expressing c-fos, c-jun, and c-myc was increased in all lobes of positive control (CCL4) animals compared to that seen in untreated and corn oil controls. In livers of CCL4 treated animals the expression of c-fos (data not shown) and c-jun (Fig. 9c) was observed in the centrilobular region of each lobe while c-myc (Fig. 9d) was expressed mainly in the centrilobular or midzonal region.

In Situ Hybridisation Analysis of Expression of IE Genes

IE gene expression in each of the lobes of individual animals within each treatment group was determined by ISH. c-fos (data not shown) and c-jun (Fig. 9a) were barely detectable in any cells in any lobe examined from the majority of untreated or corn oil control animals. Expression of c-myc in control or corn oil animals was detected in the occasional cell, mainly in the midzonal region (Fig. 9b). As seen by Northern blot analysis, the number of cells expressing c-fos, c-jun, and c-myc was increased in all lobes of positive control (CCL4) animals compared to that seen in untreated and corn oil controls. In livers of CCL4 treated animals the expression of c-fos (data not shown) and c-jun (Fig. 9c) was observed in the centrilobular region of each lobe while c-myc (Fig. 9d) was expressed mainly in the centrilobular or midzonal region.

FIG. 6. Expression of c-jun in individual animals. Northern blots were quantified by image analysis and expression normalised to GAPDH.

FIG. 7. Expression of c-myc in individual animals. Northern blots were quantified by image analysis and expression normalized to GAPDH.
 Although DCB and DEHP treatment increased the number of cells expressing c-fos and c-jun compared to control, there were fewer labeled cells than in CCl₄ treated animals. In the DCB and DEHP treated animals, the c-fos (data not shown) and c-jun (Fig. 9e, DCB; 9g, DEHP) labeled cells appeared to be randomly distributed across the lobule. In CEA treated animals there were no cells expressing detectable levels of c-fos; c-jun was expressed in an occasional cell in the midzonal region (data not shown). The number of cells expressing c-myc was increased in the DCB (Fig. 9f) and DEHP (Fig. 9h) treatment groups; as well as in one CEA treated animal (data not shown); the labeled cells were mainly midzonal although there was some perportal labeling.

**DISCUSSION**

The role of DNA synthesis in the mechanism of nongenotoxic hepatocarcinogenesis is unclear. Despite a good correlation between the ability of a nongenotoxic carcinogen to induce DNA synthesis and its subsequent hepatocarcinogenicity (Ashby et al., 1994), several noncarcinogenic chemicals also induce hepatocyte DNA synthesis. Hepatocyte proliferation is a complex process requiring a tightly controlled balance of factors. The IE genes are thought to be essential to stimulate cell proliferation (Evan and Littlewood, 1993, reviewed by Taub, 1996) and the involvement of c-fos, c-jun, and c-myc in regenerative hyperplasia has been demonstrated clearly (Coni et al., 1990; Herbst et al., 1991; Goldsworthy et al., 1994). However, the involvement of these IE genes in direct hyperplasia caused by nongenotoxic hepatocarcino-
FIG. 9. Photomicrographs of expression of c-jun in untreated (A), CCl₄ (C), DCB (E), and DEHP (G) or c-myc in untreated (B), CCl₄ (D), DCB (F), and DEHP (H) treated rat liver detected by in situ hybridization. In each field a labeled cell is indicated by an arrow. Portal vein and central vein are depicted by p or c, respectively.
DCB, which is not hepatocarcinogenic in rat (NTP, 1987b), caused an increase in LI and an induction of IE gene expression that was comparable with that induced by DEHP. As observed for DEHP, results were similar in each of the lobes examined from each individual liver but there was interanimal variation in both gene expression and LI. Thus, no differences between DEHP and DCB were detected in either the quantity of IE gene induction by Northern blotting or the localization of the expression by ISH. These data suggest that the hepatocarcinogenic liver mitogen, DEHP, cannot be distinguished from the noncarcinogenic liver mitogen, DCB, by the pattern of IE gene induction. The chronic S-phase inducer and hepatocarcinogen, CEA, did not elevate expression of the IE genes c-fos and c-jun, and in the majority of animals, c-myc; this correlated with the lack of induction of S-phase 0–48 h after dosing and with the previously observed lack of induction of S-phase in the acute (0–7 day) phase (Odum et al., 1997).

We examined the expression of all three IE genes, c-fos, c-jun, and c-myc, at a single time point, 1 h after dosing. This time point was chosen since studies in our laboratory have shown that in F344 rats maximal expression of these three genes occurred 0.5–1 h after partial hepatectomy and peak expression of c-myc and c-fos occurred within 0.5–1 h after administration of the peroxisome proliferator methylclofenapate, (Holden et al., 1994). These data agree with those from other laboratories where peak expression of c-myc in F344 rats was reported by Goldsworthy et al. (1994) 1 h after dosing and peak expression of c-fos 0.5–2 h after partial hepatectomy (Coni et al., 1990). Interestingly, there appear to be strain differences in the timing of expression of c-myc following a regenerative growth stimulus. We have shown increased expression of c-myc in F344 rats 1 h after CCl₄, which is consistent with increased expression of c-myc in F344 rats within 30 min of PH or CCl₄ (Goldsworthy et al., 1994). Peak expression of c-myc occurred later in other strains of rat, 2–3 h after PH or CCl₄ in Wistar rats (Coni et al., 1990) and the increase was not detectable until 3 h after CCl₄ in Sprague–Dawley rats (Herbst et al., 1991). It is possible that similar strain related differences may exist in the timing of IE gene expression induced by chemical mitogens.

The discrepancy between our results for IE gene expression after PPs and those of Coni et al. (1993) may be due to differences in experimental design. We have analyzed three separate lobes from three animals per group; it is possible that the IE gene induction we have detected by Northern blot analysis could have been masked by interanimal variation if we had pooled RNA from the livers of responsive and nonresponsive animals. This hypothesis is supported by the interanimal variation we have detected by ISH. The interanimal variation in IE gene expression we have reported may be true biological variation since this was consistent with the interanimal variation seen in LI and the variation in IE gene expression within the CCl₄ group. These findings emphasize the value of multiple data sets from individual animals rather than pooled samples when using Northern blot analysis of gene expression. It is likely that IE genes act as an “on/off” switch and that the small increases detected by Northern blot indicate genes turned on in a few cells. Our use of parallel sections for ISH indicated a large increase in IE gene expression in only a few cells rather than a small increase in expression level in all cells.

We have shown that for DCB, DEHP, CEA, and CCl₄, the level of expression of c-myc correlated well with subsequent LI. This correlation holds both with and without inclusion of CCl₄ which, being a strong inducer of LI, could have weighted the analysis; comparison of the correlation coefficients shows that inclusion of CCl₄ did not bias the result. These data suggest that expression of c-myc may simply reflect the magnitude of the subsequent proliferative response rather than mechanistic differences between proliferative stimuli. It seems likely that the cell population in which the IE gene expression is induced is that which subsequently will undergo DNA synthesis. It is unfortunate that such a hypothesis cannot be tested since technical limitations prevent IE gene expression being determined and correlated with subsequent LI on an individual animal; analysis of gene expression and subsequent LI is possible only on a group mean basis.

In summary, we have demonstrated that increased LI is preceded by an increase in expression of c-jun, c-fos, and c-myc in some cells from some animals treated with DCB or DEHP. In addition, there is a good correlation between expression of c-myc and LI. There is considerable interanimal variation in expression of the IE genes and in the LI, possibly explaining discrepancies between our findings and those of others. Despite the disparate carcinogenic potential of DEHP and DCB, both chemicals induced similar patterns of IE gene expression and subsequent LI, suggesting that IE gene expression does not provide a means of distinguishing direct acting hepatocarcinogenic liver mitogens from noncarcinogenic liver mitogens. Although the hypothesis behind the data presented here may be disproved, the observation that the S-phase induced by DCB carries a lower risk for subsequent hepatocarcinogenicity than that induced with DEHP remains. It is interesting to speculate on alternative hypotheses for the observed differences in hepatocarcinogenic risk. One possibility is that hepatocarcinogenic liver mitogens and noncarcinogenic liver mitogens stimulate S-phase in hepatocytes of a different ploidy/nuclearity class. Recent data from parallel experiments support this hypothesis (Hasmall and Roberts, 1997). The data presented in the present paper assist in the evaluation of IE gene expression as a marker of direct versus regenerative hyperplasia and as an indicator of the hepatocarcinogenic potential of liver mitogens.

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