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Changes in cell cycle-associated gene expression in a model of impaired liver regeneration

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Abstract

Following partial hepatectomy (PH) there is compensatory regeneration of the remnant liver which eventually restores hepatic mass and function. The response to PH was studied in normal BALB/c and athymic nude mice, a model of impaired liver regeneration. Following PH, nude mice demonstrated diminished peak hepatic [^{3}H]thymidine uptake and delayed liver mass restoration through 60 h post-PH. However, between 72–120 h there was no significant difference in mass restoration between the groups. The expression of genes associated with different stages of the cell cycle was evaluated in both models. In nude mice, there was an increase in peak expression of c-*jun* transcripts, while c-*myc* transcript expression was moderately attenuated. Thymidine kinase (TK) and cyclin-dependent kinase 1 (CDK1) mRNA expression was also diminished in athymic nude mice. The results suggest that while the defect in the regenerative response of the nude mouse after PH affects events in several phases of the cell cycle, mass restoration of the liver is only delayed and not attenuated.

Key words: Athymic nude mouse; Cell cycle; Gene expression; Liver regeneration; Proto-oncogene; Transcript

1. Introduction

Following a wide variety of acute hepatic injuries, the capacity of the liver to recover is largely due to regenerative growth. In this process, differentiated hepatocytes are induced to proliferate, resulting in the ultimate restoration of hepatic parenchymal mass. This growth response is easily studied in laboratory rodents following 2/3 partial hepatectomy (PH). In rats and mice, PH is rapidly followed by the entry of a large percentage of hepatocytes into the cell cycle and a relatively synchronous progression through DNA synthesis and mitosis. PH has therefore been used as a model for the study of eukaryotic growth control in vivo and has allowed the identification of certain hepatic growth factors and cellular genes involved in the process [1].

The involvement of growth response genes in cellular proliferation is well established but still incompletely understood. These include cellular proto-oncogenes which show increased expression at various times following growth stimulation. The importance of these genes has been documented by in vitro studies showing their induction by known growth factors, activity of their protein products in such key processes as transcription and DNA synthesis, interruption of the cell cycle by prevention of their expression, and cellular transformation or unrestricted growth induced by their over-expression [2]. Thus, the coordinated expression of these growth response genes plays a critical role in cell replication.

The study of liver regeneration has provided insight into the significance of these genes during controlled cell proliferation in the intact animal. This has included numerous studies documenting the expression of 'immediate-early' proto-oncogenes induced within minutes of PH. The increase in their expression is thought to mediate progression of hepatocytes from a differentiated and mitotically quiescent state (G_0) into the early stage of the cell cycle (G_1) [1]. In addition, genes involved with later phases of the cell cycle are also expressed following PH, often exhibiting patterns consistent with their behavior in synchronized cell culture systems [3,4]. However, the expression of growth response genes in the regenerating liver is occasionally different from that predicted by in vitro or cell culture experiments. This has been documented most extensively for certain immediate-early proto-oncogenes, such as c-*myc*, c-*jun*, and c-*fos* [5–7].

It has been previously shown that homozygous athymic nude mice exhibit a markedly depressed early regenerative response to PH compared with normal (BALB/c) mice, as measured by [^{3}H]thymidine uptake [8]. Whereas the typical murine response to PH is an abrupt 40-fold increase in remnant liver thymidine index at 30–36 h post-PH, nude mice showed a significantly altered response with a much smaller peak at 42 h. The present study extends the characterization of this unique model of impaired liver growth, and demonstrates that both [^{3}H]thymidine uptake and hepatic mass restoration are only temporarily diminished. In addition, prolifera-

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Abbreviations: CDK1, cyclin-dependent kinase 1; EGF, epidermal growth factor; LPS, lipopolysaccharide; TK, thymidine kinase; TNFα, tumor necrosis factor α.

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tion response genes associated with later stages of the cell cycle show diminished expression in nude mice, whereas immediate-early proto-oncogenes are variably affected. The results suggest that the factors leading to impaired regeneration in the athymic nude mouse may affect later cell cycle events to a greater degree than those observed during the early stages. Furthermore, they support the concept that immediate-early proto-oncogene mRNA expression does not predictably reflect proliferative activity in regenerating liver.

2. Materials and methods

2.1. Animals

Male 20–25 g BALB/c and athymic nude (*nu/nu*) mice (Harlan Sprague–Dawley, Indianapolis, IN) were maintained in specific pathogen-free housing with 12 h light/dark cycling and fed ad libitum. 2/3 partial hepatectomy (PH) was performed between 08.00 and 12.00 h as previously described [9] using ether anesthesia. Resection of the liver included the left and median lobes as well as the gall bladder. The procedure effectively removed 65 ± 5% (mean ± S.D.) of total liver mass. Surgical and post-operative mortality was less than 5% and did not differ between the BALB/c and athymic nude mice. At various times post-PH, mice were sacrificed and the remnant liver was removed and flash-frozen in liquid nitrogen. In selected animals, salivary glands were excised as previously described [10]. Neonatal thymectomy was performed on BALB/c mice by the commercial vendor, and the animals subjected to PH as described above.

2.2. [^{3}H]Thymidine incorporation and mass restoration

DNA synthesis was determined after intraperitoneal injection of 0.4 μCi/g b.wt. [^{3}H]thymidine (20–30 Ci/mmol; Amersham, Arlington Heights, IL) 1 h prior to sacrifice. Homogenates of frozen liver were prepared as previously described [11] and extracts were subjected to liquid scintillation counting and colorimetric DNA assay [12]. Mass restoration was estimated by weighing the resected liver segment and the remnant liver tissue of each animal at the indicated times. The percentage of regenerated mass after PH was calculated as: [actual weight of liver remnant − expected weight] ÷ prehepatectomy liver weight × 100, where expected weight of liver remnant was 35/100 × the prehepatectomy liver weight.

2.3. Salivary gland EGF assays

Salivary glands from control 0 h BALB/c and athymic nude mice were homogenized and assayed for EGF using a commercial kit and the manufacturer's recommendations (Biomedical Technologies Inc., Stoughton, MA).

2.4. RNA preparation and Northern blot analysis

Total RNA was prepared from the pooled liver remnants of 2–5 mice using guanidium isothiocyanate [13]. Poly(A)$^+$-enriched RNA was obtained by oligo(dT) chromatography and quantitated spectrophotometrically at 260 nm absorbance. Northern blot analysis of poly(A)$^+$-enriched RNA (5 μg/lane) was performed as previously described [14]. Membranes were hybridized with the cDNA probes listed below for 24–30 h at 42°C in buffer containing 50% formamide and 0.2% SDS. Conditions for the preparation and analysis of the Northern blots for RNA samples from the BALB/c and athymic nude mice were identical, including electrophoresis and transfer, cDNA probe specific activity and hybridizations, washings and autoradiography.

The following cDNA probes were used: a 0.5 kb *Pst*I fragment of rat albumin [15]; a 1.08 kb *Eco*RI fragment of mouse CDK1(p34^{cdc2}) [16]; a 0.45 kb *Sma*I–*Sal*I fragment of mouse H-*ras* [17]; a 1.4 kb *Eco*RI fragment of human c-*jun* [18]; a 2.2 kb *Eco*RV–*Hin*dIII fragment of mouse c-*myc* [19]; a 1.47 kb *Eco*RI fragment of rat p53 [20], and a 1.2 kb *Bam*HI fragment of mouse thymidine kinase [21]. Probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by random priming [22]. Following hybridization, membranes were washed and exposed to Kodak XAR film at −70°C for 1–7 days using an intensifying screen. After probing, the membranes were stripped and rehybridized with the albumin cDNA. Autoradiograms were analyzed by video-densitometry as previously described [14]. For quantitation, band densities were normalized to the invariant expression of the albumin mRNA [23].

3. Results

3.1. [^{3}H]Thymidine incorporation, mass restoration and EGF levels

Previous studies of the regenerative response in athymic nude mice evaluated only the thymidine index during the first 45 h post-PH [8]. In the present study, both [^{3}H]thymidine incorporation and mass restoration were determined over 120 h following the procedure. The results demonstrated that there was a peak in [^{3}H]thymidine uptake at 36 h in the BALB/c mice (Fig. 1A), which is consistent with results previously reported [8]. In the athymic nude mice, [^{3}H]thymidine uptake was significantly reduced at 36 h, and showed a broad but relatively diminished peak at later time points. From 48–120 h, DNA synthesis was increased relative to control (0 h) animals, but was not significantly different in the two groups. By 120 h post-PH, there was a marked decline in thymidine index in both groups. To test whether the absence of the thymus gland altered liver regeneration, 4 BALB/c mice underwent surgical thymectomy shortly after birth. After growing to the appropriate size, they were subjected to PH and showed a [^{3}H]thymidine uptake of 68.1 ± 29.1 cpm/μg DNA at 36 h, which more closely resembled the uptake by athymic nude mice than by that of BALB/c mice. The relative mass restoration following PH is shown in Fig. 1B. In support of the [^{3}H]thymidine uptake data, there was diminished mass restoration from 24–60 h in the athymic nude mice. However, at 72, 96 and 120 h there was no significant difference in mass restoration between the two groups.

EGF is thought to play a major role in the process of liver regeneration, and the salivary glands are the major source of EGF in rodents [1,10]. We determined whether the altered regenerative response in athymic nude mice was mediated by diminished salivary production of the hormone. Salivary gland EGF levels were measured and were found to be no different in the BALB/c and nude mice (883 ± 63.5 and 818 ± 63.1 ng/mg wet weight, respectively).

3.2 Expression of growth response genes

The expression of six different cell cycle-associated genes was evaluated in the regenerating livers of athymic nude and BALB/c mice. As shown in Fig. 2, there was an abrupt rise in c-*myc* mRNA expression within 30 min after PH in BALB/c mice exhibiting a peak at 1 h and continued elevations at 3 and 6 h. In nude mice, there was a similar induction although the peak values from 1–6 h were moderately diminished. The c-*jun* transcripts

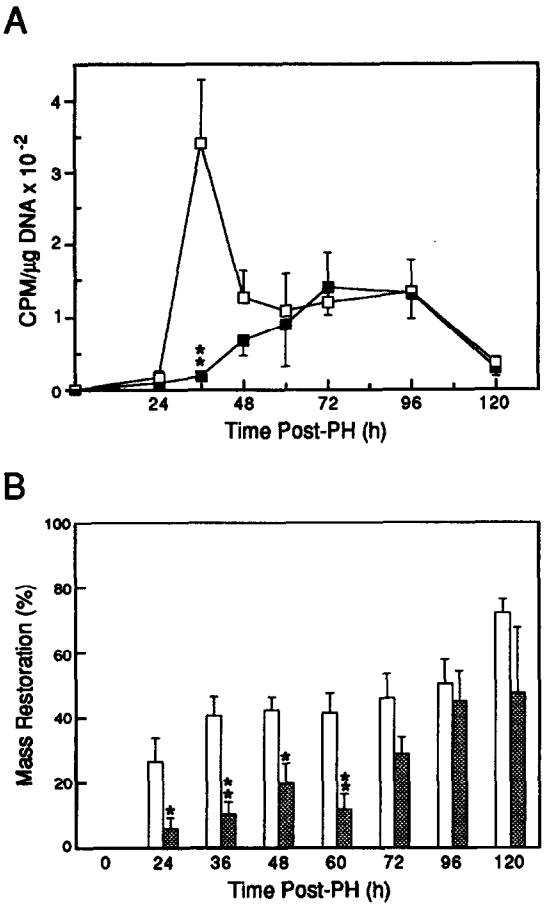

Fig. 1. Liver regeneration in BALB/c (□) and athymic nude mice (■). (A) [^{3}H]Thymidine incorporation after 65% PH. Mice were injected intraperitoneally with 0.4 μCi/g b.wt. [^{3}H]thymidine 1 h prior to sacrifice at the time points indicated. DNA was extracted from the remnant livers and [^{3}H]thymidine uptake determined as described in section 2. Values are expressed in cpm/μg DNA as the mean ± S.E.M. of at least 4 determinations per group. (B) Liver mass restoration after PH. Remnant liver weights were used to calculate percent mass restoration as described in section 2. Values are expressed as the mean ± S.E.M. of at least 4 determinations at each time point. $^*P < 0.05$ and $^{**}P < 0.001$ compared with BALB/c control group.

were also dramatically induced shortly after PH. The initial peak of c-*jun* expression occurred at 1 h in both groups, with a more pronounced peak in the nude mice. At 3–12 h after PH, the expression of c-*jun* was nearly equivalent in the two groups. The patterns of c-*myc* and c-*jun* induction in the BALB/c mice were similar to those previously reported [24].

The proto-oncogene H-*ras* and p53 tumor suppressor gene are critical effectors of the cell cycle and appear to be associated with events in G_1 phase [2]. Both transcripts showed only moderate induction at 12–24 h after PH (Fig. 3), and the patterns of expression were indistinguishable between the BALB/c and nude mice. The thymidine kinase (TK) gene is significantly induced during active DNA synthesis (S phase) in cultured cells [25]. As shown in Fig. 4, there was low basal expression of this gene in control liver (0 h) exhibiting a decline at 12–24 h post-PH. In the BALB/c mice, this was followed by a marked increase in TK transcript expression peaking at 60 h. In contrast, nude mice exhibited relatively blunted expression of TK after PH. For comparison, expression of the CDK1 gene, which is involved in the control of M phase, was also examined (Fig. 4). As reported previously, its transcript showed peak expression at 60 h in the BALB/c mice, with delayed and diminished expression in the nude mouse [14].

4. Discussion

Regenerative hyperplasia of the liver following PH is an important model of physiologic cell growth in the intact animal [1]. It has been used extensively to examine the expression of cell cycle-associated genes, and provides a relatively unique in vivo setting to corroborate findings of in vitro systems. Furthermore, the process of liver regeneration is of great relevance to the recovery from acute liver disease in humans and animals [26]. This study was undertaken to compare several aspects of the regenerative response in normal BALB/c and athymic nude mice.

The initial characterization of the defective regenerative response in the nude mouse demonstrated markedly diminished hepatic [^{3}H]thymidine uptake during the first 45 h post-PH [8]. The results presented here confirm that the peak [^{3}H]thymidine uptake at 36 h post-PH observed in the regenerating liver of BALB/c mice is absent in nude mice. The initial [^{3}H]thymidine peak is thought to represent S phase in a population of hepatocytes progressing through the cell cycle in a relatively synchronous manner [1]. However, the lack of difference in thymidine index between BALB/c and nude mice at later time points suggests that despite the initial delay in DNA synthesis, cell replication in the athymic mouse eventually occurs. This is supported by the mass restoration data, indicating that although the regenerative process in nude mice lagged behind their BALB/c counterparts 24–60 h post-PH, no significant differences were identified at 72–120 h.

The mechanisms which control hepatic regeneration are incompletely understood, but are thought to include numerous growth factors derived from the liver and extrahepatic sites [1]. The remarkable absence of the initial 36 h [^{3}H]thymidine peak in the nude mouse suggests the possible lack of mitogenic stimuli and/or the presence of growth inhibitors. It has been previously demonstrated that salivectomy, which results in diminished systemic EGF levels, produces delayed liver regeneration in mice following PH [10]. However, the present results indicate that salivary gland production of EGF is no different between control BALB/c and athymic nude mice and does not appear to account for the observed differences in regenerative response.

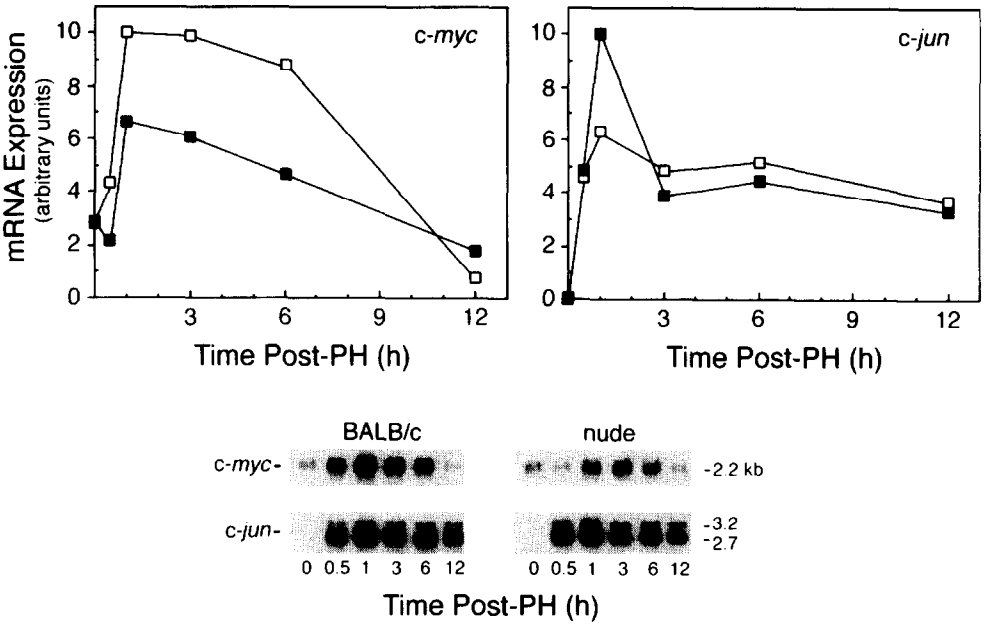

Fig. 2. Expression of c-*myc* and c-*jun* mRNA steady-state transcripts in the livers of BALB/c (□) and athymic nude (■) mice after PH. Poly(A)$^+$-enriched RNA (5 μg/lane) was isolated from remnant livers and subjected to Northern blot analysis as described in section 2. Autoradiograms were quantitated by video-densitometry and normalized to the expression of the albumin mRNA transcript. The figures are representative of 3 separate determinations. The values at each time point are expressed relative to 10 arbitrary units of mRNA expression. Transcript sizes are indicated at the right.

It was originally hypothesized that the diminished regeneration by nude mice was due to the absence of lymphocyte-derived cytokines, which are normally stimulated by the presence of gut-derived lipopolysacharride (LPS) [8]. This concept was supported by the observation that germ-free euthymic and LPS-resistant mice also demonstrated delayed [^{3}H]thymidine uptake after PH, although to a lesser extent than the athymic nude mice. Additional studies have documented that numerous cytokines can affect hepatocyte proliferation, although the effect is frequently inhibitory [27]. Tumor necrosis factor α (TNFα) has been shown to be mitogenic for liver cells. Pre-treatment with antibody to TNFα diminishes [^{3}H]thymidine uptake after PH in rats [28]. It is conceivable that diminished release of TNFα, or other cytokines, due to the absent thymus in the nude mouse contributes to the impaired initial regenerative response. This is supported by our finding that thymectomized BALB/c mice showed reduced [^{3}H]thymidine uptake at 36 h post-PH relative to the non-thymectomized group. Alternatively, the genetic defect in the nude mice, which leads to several phenotypic abnormalities, may affect liver regeneration by mechanisms which are independent of the immune response.

Expression of the immediate-early genes following PH or acute hepatotoxin exposure in rodents is well documented [1]. Within 30 min after PH, there is both transcriptional and post-transcriptional induction of c-*myc*, c-*jun*, and c-*fos* mRNA expression in rats and mice. While their precise role in hepatic regeneration is not understood, these immediate-early genes are thought to be critically involved in the progression of liver cells through the cell cycle from G_0 to G_1 after PH [29]. However, the relationship of these genes to hepatocyte replication is dependent upon the nature of the proliferative stimulus. For example, it has recently been demonstrated that hepatocyte proliferation induced by cyproterone acetate and nafenopin is not associated with increased expression of c-*myc*, c-*jun*, or c-*fos* [5]. In contrast, dietary manipulation can induce expression of these immediate-early genes in the absence of cellular replication [6]. It has been hypothesized that these proto-oncogenes regulate a prereplicative 'priming' phase for hepatocytes after PH and that 'progression' through the cell cycle is dependent upon additional factors and the expression of genes gov-

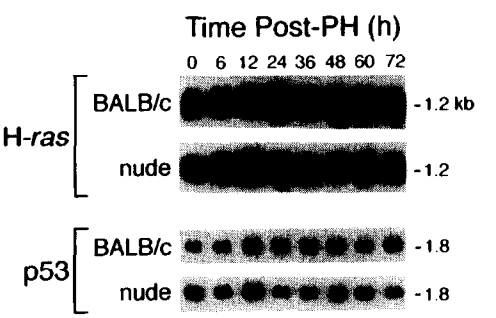

Fig. 3. Expression of H-*ras* and p53 mRNA steady-state levels after PH in BALB/c and athymic nude mice. Northern blot analysis was performed as described in Fig. 2. The figures are representative of 3 separate determinations. Transcript sizes are indicated at the right.

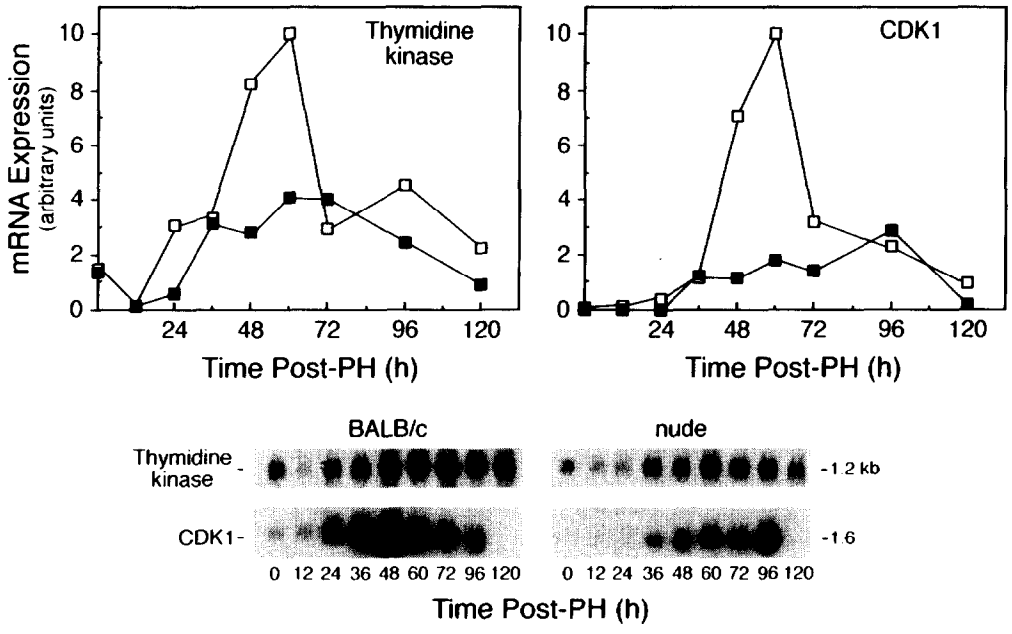

Fig. 4. Thymidine kinase and CDK1 mRNA transcript expression after PH in BALB/c (□) and athymic nude (■) mice. Northern blot hybridizations and quantitation by video-densitometry were performed as described in Fig. 2. The figures are representative of 3 separate determinations. Transcript sizes are indicated at right.

erning events later in the cell cycle [1,29]. In BALB/c and athymic nude mice, there was rapid induction of both c-*jun* and c-*myc* mRNA after PH, suggesting that the stimuli initiating immediate-early gene expression after PH are not absent in the nude mice. However, the magnitude of peak c-*myc* expression in the athymic mouse was moderately diminished. Conversely, peak c-*jun* expression was greater in the nude mouse, indicating that the signals modulating the expression of this transcription factor are intact. The observed expression of c-*myc* and c-*jun* in the nude mouse contrasts with the absence of the 36 h [^{3}H]thymidine uptake peak following PH. The results suggest that the initial priming of hepatocytes during the cell cycle is intact, and that the defective regenerative response is modulated by events which occur later in the cell cycle. However, it is conceivable that the functional expression of the c-*myc* and c-*jun* proteins in the nude mouse is abnormal and no longer reflective of their respective transcript levels.

Expression of the p53 and H-*ras* genes was evaluated in the two models to examine potential differences in the G_1–G_2 phase of the cell cycle. Both genes demonstrated only moderate induction of their respective mRNA transcripts after PH; no significant difference in expression was observed between the two groups. In contrast, expression of TK and CDK1 in the mouse models was remarkable for two reasons. First, peak expression of the genes occurred at 60 h post-PH in the BALB/c mouse which was significantly later than the observed [^{3}H]thymidine peak at 36 h. This was somewhat surprising since TK and CDK1 are known to be active in S and M phases, respectively. However, it is possible that their proteins peak earlier than their respective transcripts. In this regard, disparate expressions of mRNA and associated proteins have been demonstrated for other cell cycle-associated genes including cyclin D1 [30]. Alternatively, [^{3}H]thymidine uptake may not always be an accurate indicator of S phase [31]. Second, the expression of both transcripts was notably diminished at 48–60 h in nude mice, suggesting that gene expression during the later stages of the cell cycle is delayed in these animals. The observation suggests that genes expressed late in the hepatocyte cell cycle may more accurately reflect hepatic regenerative activity than immediate-early genes [5,14]. This may have relevance to the study of human liver diseases, where cell cycle gene expression has been used as a surrogate marker of hepatic regenerative activity [32].

In conclusion, the results of this study confirm that athymic nude mice demonstrate a diminished early regenerative response following PH, but hepatic DNA synthesis and growth of the remnant liver mass does eventually occur. The nature of the defect causing impaired liver regeneration is unknown, but it appears to affect late cell cycle events more prominently than the priming response mediated by c-*jun* and c-*myc*. Expression of these immediate-early proto-oncogene transcripts is not always tightly linked to the extent of liver regeneration.

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