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## **Id2 leaves the chromatin of the E2F4-p130-controlled *c-myc* promoter during hepatocyte priming for liver regeneration**

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Short title: "Id2 and priming for liver regeneration".

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## ABSTRACT

The Id helix-loop-helix proteins are involved in the regulation of cell growth, differentiation and cancer. The fact that the molecular mechanisms of liver regeneration are not completely understood prompted us to study the fate of Id2 in proliferating liver. Id2 increases in liver regeneration after partial hepatectomy, following the early induction of its gene. Co-immunoprecipitation shows that Id2 forms a complex with E2F4, p130 and mSin3A in quiescent liver and all these components are present at the *c-myc* promoter as shown by chromatin immunoprecipitation (ChIP). Activation of *c-myc* during hepatocyte priming ( $G_0$ – $G_1$  transition) correlates with the dissociation of Id2 and HDAC, albeit p130 remains bound at least until 6 h. Moreover, as the transition  $G_0$ – $G_1$  progresses, Id2 and HDAC again bind the *c-myc* promoter concomitantly with the repression of this gene. The time course of *c-myc* binding to the *Id2* promoter, as determined by ChIP assays is compatible with a role of the oncoprotein as a transcriptional inducer of *Id2* in liver regeneration. Immunohistochemical analysis shows that Id2 also increases in proliferating hepatocytes after bile duct ligation. In this case, the pattern of Id2 presence in the *c-myc* promoter parallels that found in regenerating liver. Our results may suggest a control role for Id2 in hepatocyte priming, through a p130 dissociation-independent regulation of *c-myc*.

## INTRODUCTION

The Id (inhibitor of DNA binding or inhibitor of differentiation) helix-loop helix (HLH) protein family plays a central role in the regulation of cell growth and differentiation in many mammalian tissues [1, 2]. Four members of this family, Id1 to Id4, have been identified. The first role described for them was their binding to another group of basic helix-loop-helix (bHLH) transcription factors, blocking their association with DNA [3, 4]. While this association may result in the inhibition of cell differentiation (reviewed in 5, 6), the roles of Id proteins in regulating cell growth are far more complicated than originally thought. Id proteins also associate with other factors involved in cell cycle regulation, for instance the members of the pRb family, key factors in the control of the G<sub>1</sub>-S phase transition.

Several tumour cell lines, as well as some primary human tumours, have been shown to express *Id* genes in a deregulated manner [7]. However, Id2, but not Id1 and Id3, associate with the hypophosphorylated forms of the pocket proteins and disrupt their antiproliferative effects [8]. To further investigate the role of Id2 proteins in cell proliferation, the selection of an appropriate biological model is of crucial importance. Liver regeneration is a highly regulated process of tissue repair and replacement involving proliferation of different liver cell populations [9, 10]. This process, involved in the response and defence against toxic insults or viral infections, is of outstanding importance because of the capacity of the liver to regulate its own growth and mass and it plays a crucial role in degenerative processes such as liver cirrhosis [9, 11]. Liver regeneration can be experimentally induced by partial hepatectomy (PH), the surgical removal of ~70% of the liver [12]. This triggers the proliferation of the remaining parenchyma cells that divide once or twice, to quickly restore the liver mass prior to returning to quiescence [9, 10]. This behaviour is possible because the hepatocytes have a very high replication capacity, comparable to or exceeding that of precursor cells of proliferating tissues [13]. Much effort has been dedicated to understand the molecular events that trigger liver regeneration. The role of growth factors in the mitogenic response of hepatocytes after PH has been clearly established [9, 14]. Nevertheless, quiescent hepatocytes in normal liver do not respond to proliferating stimuli unless primed [15]. It is accepted that priming corresponds to the G<sub>0</sub>-G<sub>1</sub> transition [9]. The ultimate molecular mechanisms responsible for this process is not yet fully understood, although using a microarray methodology, Locker *et al.* [16] have identified 54 immediate-early genes up-regulated during priming.

In the present work, we analyze the expression of *Id2* gene in regenerating rat liver after

PH. As one of the genes that are under the control of pocket proteins is *c-myc*, we also investigated the possible relation between Id2 and hepatic *c-myc* induction. The fact that *c-myc*, an essential regulator of the G<sub>0</sub>–G<sub>1</sub> transition [17, 18], is one of the immediate-early genes induced after PH [9, 16] adds an additional interest to this study. Our results suggest a role for Id2 protein in the control of hepatocyte priming and provide new insights into the chromatin-associated mechanisms involved in the induction of *c-myc* promoter during liver regeneration.

## EXPERIMENTAL

### Animals

Male, pathogen-free Wistar rats (220-260 g) were held in groups of two in macrocol cages at 22 °C with a 12 h light-dark cycle and fed *ad libitum* with free access to water. Animals were cared and handled in conformance with the “Current Protocols for Protection of Animals Used for Experimental and Other Scientific Purposes” (Council Directive 86/609/EEC). For partial hepatectomy (PH) the two anterior lobes were removed under anaesthesia according to Higgins and Anderson [12] and for BDL model, animals were bile duct ligated according to Kontouras *et al.* [19]. Animals were sacrificed 28 days after surgery under anaesthesia and the liver was quickly removed. The study was approved by the Research Committee of the University of Valencia.

### RT-PCR studies

Total RNA from rat liver was isolated by guanidinium thiocyanate method [20]. Aliquots of 2 µg were reverse-transcribed using SuperScript II (GibcoBRL) and subsequently amplified by polymerase chain reaction (PCR) using AmpliTaq DNA polymerase (Applied Biosystems). The primers used for the different genes studied were: 5'-ACGAGCAGCATGAAAGCCTT-3' and 5'-AGGATGCTGATGTCCGTGTTC-3' for *Id2*; 5'-ACCTCGACTACGACTCGGTG -3' and 5'-AGAAGCCGCTCCACATACAG-3' for *c-myc*. 18S rRNA was simultaneously amplified using the QuantumRNA 18S Internal Standards (Ambion) as internal loading control. Reactions were resolved in a 2% agarose gel, stained with ethidium bromide and quantified using the GeneGenius System and the Gene Tools analysis software (Syngene).

Real-time quantitative PCR was performed using double-stranded DNA binding dye Syber Green PCR Master mix in an ABI GeneAmp 7000 Sequence Detection System (Applied Biosystems). Each reaction was run in triplicate for two series of animals and melting curves were constructed, using Dissociation Curves Software (Applied Biosystems), to ensure that only a single product was amplified. As a quantitative control, 18S rRNA was also analyzed.

### **Immunoblot Analysis**

Liver was homogenized in 10 ml of ice-cold RIPA buffer (1.85 mM NaH<sub>2</sub>PO<sub>4</sub>, 8.4 mM Na<sub>2</sub>HPO<sub>4</sub> pH 7.9, 0.1 M NaCl, 12 mM deoxycholate, 0.1% SDS, 1% Triton X-100, 0.5% NaN<sub>3</sub>) per gram of tissue in the presence of protease inhibitors (Sigma). The homogenate was centrifuged at 160,000 *g*. Equal amounts of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Id2 was detected by using the corresponding antibody (Santa Cruz sc-489) and secondary horseradish peroxidase-conjugated antibody. Blots were developed by enhanced chemiluminescence (DuPont). The loading of the gels was checked with an antibody specific for  $\beta$ -actin.

### **Immunohistochemical detection of Id2**

Sections (5  $\mu$ m thick) of paraffin embedded liver were hydrated and pre-treated with EDTA pH 8. Sections were incubated with 3% H<sub>2</sub>O<sub>2</sub> for 20 min to inhibit endogenous peroxidase and then with swine normal serum (10% in PBS and 0.1% BSA) for 30 min. Incubation with primary antibody (Zymed Laboratories) was performed overnight at 4°C (diluted 1:500). Sections were incubated with secondary antibody, swine anti-rabbit (DakoCytomation, Denmark), diluted 1:200 for 1 h at room temperature, and then with a visualization reagent (ABCComplex/HRP, DakoCytomation, Denmark) for 25 min. Positive reaction was evidenced with diaminobenzidine.

### **Co-immunoprecipitation**

Liver tissues (2 g) were washed twice with 10 ml of cold PBS pH 7.4 and immersed into 8 ml of PBS supplemented with 2  $\mu$ l/ml of protease inhibitor and 2  $\mu$ l/ml each of phosphatase inhibitor cocktails I and II (Sigma). Tissues were disrupted with a Dounce homogenizer and centrifuged at 1500 *g* for 5 min. The pellets were resuspended in 3 ml of cell lysis buffer (5 mM HEPES pH 8.0, 85 mM KCl, 0.5% NP40) supplemented with protease and phosphatase inhibitors as above, incubated on ice for 15 min and centrifuged at 3500 *g* for 5 min to pellet the nuclei. The nuclei were resuspended in 3 ml nuclear lysis buffer (1 $\times$ PBS, pH 7.4, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with the above-mentioned protease and phosphatase inhibitor cocktails. The nuclear extract was fractionated into aliquots containing 1 mg protein each.

Co-immunoprecipitation was performed by adding 4  $\mu$ g of the corresponding antibodies

(Santa Cruz, Id2 sc-489, E2F4 sc-866, p130 sc-317, and mSin3A sc-994) to each aliquot and incubating overnight at 4 °C in a rotating plate. Controls with a non-related antibody (against *A. thaliana* amidase, gently provided by G. Ríos) and with no antibody were carried out.

Immunocomplexes were captured with protein IP matrix (Santa Cruz, ExactaCruz F sc-45043) for 4h and the pellets were washed three times in protease and phosphatase inhibitor-supplemented nuclear lysis buffer. Protein complexes were eluted in Laemmli's loading buffer and subjected to Western blotting analysis using HRP-conjugated-antibody probe (Santa Cruz, ExactaCruz F sc-45043) and ECL advance Western blotting detection kit (Amersham Bioscience).

### **Chromatin Immunoprecipitation assay and RNAPol-ChIP**

Cross-linking of chromatin, ChIP and RNAPol-ChIP procedures were performed according to Sandoval *et al.* [21]. Briefly, isolated nuclei from formaldehyde-crosslinked livers were lysed and crosslinked chromatin was sonicated to yield fragments of ~500 bp. Diluted soluble chromatin fragments were precleared with blocked protein A/G Sepharose, to discard non-specifically-bound chromatin fragments. Immunofractionation of complexes was carried out by adding 2 µg of the corresponding antibodies (Santa Cruz, Id2 sc-489, E2F4 sc-866, P130 sc-317, mSin3A sc-994, RNAPol II sc-899 and c-myc sc-764) to aliquots containing 50 µg DNA each. The immunocomplexes were recovered by centrifugation after adding blocked protein A/G Sepharose and extensively washed. Immunoselected chromatin was eluted and the formaldehyde crosslinking was reverted. The DNA from all samples was purified with a PCR purification kit (Qiagen), and used for PCR analysis, which was done with the following primers: *c-myc* (promoter) 5'-GTGCCCTCCCGAGTTCC-3' and 5'-GCGACTCCGGATCCCTCC-3'; *c-myc* (coding region) 5'-ACAACCGCAAATGCTCCAGC-3' and 5'-TCCGGTCAGTTTATGCACCAAG-3'; *Id2* (promoter) 5'-ACGGGCATTGGCTGCGAACG-3' and 5'-GAGGAAAGGCCGGGAGGGAG-3'; *Id2* (coding region) 5'-ACAAGAAGGTGACCAAGATGGAA-3' and 5'-GCGATCTGCAGGTCCAAGAT-3'; *α-actin* (promoter) 5'-AGGGACTCTAGTGCCCAACACC-3' and 5'-CCCACCTCCACCCTACCTGC-3'; *α-actin* (coding region) 5'-AGGATTCTACGTGGGCGAC-3' and 5'-TAGAGAGACAGCACCGCCTG-3'; *β-actin* (coding region) 5'-TTCAACACCCAGCCATGT-3' and 5'-GTGGTACGACCAGAGGCATACA-3'.

## RESULTS

### **Id2 gene is up-regulated during liver regeneration**

The expression of *Id2* gene was first evaluated by both, semiquantitative and quantitative RT-PCR analyses. The steady-state levels of *Id2* mRNA were significantly increased early after PH, reaching a maximum at about 1 h and returning to control values by 6 h (Figure 1A). This peak of *Id2* expression occurs during the hepatocyte priming phase (G<sub>0</sub>-G<sub>1</sub> transition). A second, yet flatter, peak of *Id2* expression occurs later during regeneration. In accordance with the results of other workers (see, for instance, 22), *c-myc*, apart of being an immediate-early gene [9], displays an expression pattern similar to that of *Id2* and its expression is resumed during G<sub>1</sub>-S transition (Figure 1B). Western blot experiments demonstrate that the level of Id2, which is low in quiescent cells, increase soon after PH, following the activation of *Id2* gene transcription (data not shown).

### **Id2 is present in the *c-myc* promoter in quiescent cells**

Id2 is able to bind to pocket proteins regulating their function [23, 24]. Specifically, the low amount of Id2 found in quiescent fibroblasts is mainly bound to p130 [25]. The co-immunoprecipitation experiment of Fig. 2 demonstrated that this binding also occur in quiescent liver cells, where p130 and Id2 form a complex with E2F4 and mSin3A (an important component of a histone deacetylase repressor complex). Massagué and his colleagues have found that complexes between E2F4/5 and the pocket protein p107 are essential for *c-myc* repression in keratinocytes [26]. As the pocket proteins inhibit the action of E2F by recruiting histone deacetylases [27-29], a mechanism also involved in placing keratinocytes in a quiescent state [30], the hypothesis that Id2 is involved in liver proliferation by regulating *c-myc* gene expression through a histone acetylation-dependent mechanism was a plausible one. To check the potential role of these factors in the regulation of *c-myc* promoter, we carried out the ChIP assay shown in Figure 3. Id2 is present in the *c-myc* promoter in quiescent cells, together with E2F4, p130 and mSin3A (Fig. 3A). It is worth noting that, to the best of our believe, this is the first time that Id2 is shown to be bound to DNA, although, most likely, on the basis of the current knowledge, the binding was not a direct one. The same samples employed for the ChIP assay were used for RNAPol ChIP [21]. Briefly, this technique consists in using the chromatin immunoprecipitated with an RNAPol II antibody for the PCR detection of the coding region of the desired gene. Therefore, it provides an excellent internal control of real time transcription. Thirty minutes after PH, *c-myc* is actively transcribed (Fig. 3C) and at this time Id2 and mSin3A dissociated from the *c-myc* promoter (Fig. 3A). A further comparison of panels A and C of Fig. 3 reveals that 6 h after



PH, when the transcription of *c-myc* passes through a minimum, *Id2* and mSin3A again appear bound to the promoter. The p130-E2F4 complex remained attached along the whole priming period, independently of the transcriptional state of *c-myc*.

### **The c-myc oncoprotein is present in the rat *Id2* promoter during hepatocyte priming**

Lasorella *et al.* [25] reported that the overexpression of *Id2* in human neuroblastoma cells is associated with the presence of extra copies of *c-myc* and that *c-myc* and N-myc activate the expression of *Id2* in fibroblasts and neuroblastoma cells, respectively. Moreover, *c-myc* directly induces the expression of *Id2* in fibroblasts by binding to its consensus sequences in the promoter of the human gene. To ascertain whether *c-myc* could also upregulate *Id2* during liver regeneration, we searched for *c-myc* consensus binding sequences in the rat *Id2* promoter to find that it contains a cluster of E-boxes susceptible to bind *c-myc*, as the human and mouse promoters do [25]. In quiescent liver, *c-myc* is not substantially bound to *Id2* promoter, but the oncoprotein is recruited soon after PH (Figure 4A). Its presence is clear at 30 min and passes through a maximum at 1 h, concomitantly with the early activation of *Id2* gene expression (compare Figs. 4A and 4C). Interestingly, RNA polymerase II is bound to *Id2* promoter even in control liver, when active transcription has not yet started (Fig. 4B). This shows that *Id2* gene ranks among those in which RNA polymerase is paused at the promoter, waiting for the signal to initiate transcription. On the other hand, comparison of the RNAPol ChIP analyses of Figs. 3C and 4C shows that transcription of *Id2* is somewhat delayed relative to the transcription of *c-myc*.

### ***Id2* is up-regulated in bile duct ligation (BDL)**

Cirrhosis, which is often regarded as a precancerous condition [31-33], shares some mechanistic aspects with liver regeneration. This fact, together with the *Id2* overexpression described in different primary human tumours [8], led us to evaluate the expression of *Id2* gene in another model of hepatocyte proliferation, namely BDL rats, which are often regarded as an experimental model of cirrhosis [19]. After 28 days of BDL, the steady-state levels of *Id2* mRNA, determined by RT-PCR, were significantly increased in BDL rat liver (Fig. 5A), as those of *c-myc* are (Fig. 5B). The *Id2* mRNA was actually translated in liver, as confirmed by Western blot (Fig. 5C). Moreover, immunohistochemical analysis allowed us to confirm that *Id2* actually accumulates not only in bile duct cells, but also in hepatocytes (Fig. 5D and E), where it localizes both to nuclei and cytoplasm. PCNA analyses showed that a significant proportion of hepatocytes are proliferating in BDL rats (data not shown). These results validate the model used in our experiments.

Finally, we studied the binding of factors to *c-myc* promoter in BDL-rats. Figure 6A shows that Id2 and mSin3A are absent from *c-myc* promoter, while E2F4 and p130 are present, in a manner similar to that observed in hepatocyte priming. This situation correlated with the presence of RNAPol II in the coding region of *c-myc* (Fig. 6B). Therefore, many Id2-related characteristics are shared by proliferating hepatocytes, irrespective of the cause that triggers proliferation.

## DISCUSSION

The events that trigger the proliferation of the quiescent hepatocytes after PH are complex and still remain unknown in many aspects. The simple infusion of growth factors such as TGF- $\alpha$  or EGF into the normal liver does not result in increased DNA synthesis, which actually takes place if the infusion is preceded by PH [34]. Therefore, hepatocyte proliferation *in vivo* requires an initial priming stimulus [9], such as PH, which allows the cells to exit from G<sub>0</sub>. Although their molecular aspects are not yet fully understood, it is known that hepatocyte priming is marked by the induction of *c-myc* gene expression [35].

Our results show for the first time that *Id2* gene expression is up-regulated in rats immediately after PH. Togo *et al.* [36], working with DNA microarrays, have recently reported a discrete increase of *Id2* transcripts 6 h after PH in mice. Nevertheless, both the quantitative PCR of Fig. 1A and the RNAPol ChIP of Fig. 4C clearly show that the level of *Id2* transcription in rats reaches a maximum at about 1 h after PH. To date, the central role assigned to Id2 in promoting dedifferentiation and cell proliferation consists in the reversal of the pRb-mediated cell cycle arrest [23], which in our model would allow the hepatocyte to progress from G<sub>1</sub> to S. However, the data presented here suggest another earlier role for Id2 in liver regeneration, namely in the hepatocyte priming, or G<sub>0</sub>–G<sub>1</sub> transition.

The promoter of *Id2* is a target for *c-myc* in many cell types [37–40] and Lasorella *et al.* [25] have shown that *c-myc* binding activates *Id2* in fibroblasts. The timing of *c-myc* binding to the *Id2* promoter (Fig. 4) also agrees with this activating role in regenerating liver, especially during hepatocyte priming. Interestingly, the ChIP experiments of Figure 3 show that Id2 protein, bound to *c-myc* promoter in quiescent hepatocytes, leaves it when the gene is expressed. This removal also occurs during hepatocyte priming, concomitantly with the parting of the histone deacetylase complex, and does not result in the displacement of p130 (Fig. 3). It is well established that the sequestering of the non-phosphorylated form of the pocket proteins by Id2 results in the progression from G<sub>1</sub> to S-phase [41]. This action of Id2 is possible because a second peak of *Id2* expression occurs at late G<sub>1</sub>, for instance, in fibroblasts [42]. During liver regeneration

there is also a second peak of *Id2* expression at 24 h (Figs. 1A and 4C), which may be involved in the G<sub>1</sub>-S progression, but the earlier events observed here are markedly different. In a recent work, Russell *et al.* [43] have shown that *Id2* prevents tumour formation in the intestinal epithelium. This result would be explained if *Id2* actually were inhibitory of the expression of *c-myc in vivo*, as our results might indicate. If it were, this unexpected link between *Id2* and *c-myc* would establish an autoregulatory loop between both proteins that may contribute to the exquisite regulation of liver proliferation.

The binding of *Id2* to *c-myc* promoter is probably indirect as it seems to be recruited to the E2F site through p130. The simultaneous presence of all these factors (Fig. 3) shows that binding of E2F and *Id2* to pocket proteins is, most likely, not mutually exclusive. On the other hand, the binding of *Id2* seems to depend on factors other than its concentration, as the pattern of promoter binding (Fig. 3A) does not follow the *Id2* expression (Fig. 1A). The lack of correlation between *Id2* concentration and binding to *c-myc* promoter also occur in BDL rats (compare Figs. 5D and 6A). It should be kept in mind that the low amounts of *Id2* present in quiescent liver are complexed with p130 (Fig. 2), as it occurs in quiescent fibroblasts [25] and that the complex containing these components, together with E2F4 and mSin3A, is bound to the *c-myc* promoter (Fig. 3). Interestingly, the early induction of *c-myc* during hepatocyte priming occurs concomitantly with the removal of both, *Id2* and the histone deacetylase component mSin3A, while E2F4 and p130 remain bound to the promoter (Fig. 3). So, the role of *Id2* protein in hepatocyte priming seems to be independent of p130 removal, in contrast with its later role in the G<sub>1</sub>-S transition [41].

Therefore, the control of *c-myc* transcription during priming, is independent of pocket protein displacement, though may depend on chromatin structure, because activation is concomitant with the removal of the deacetylase complex. The mechanism by which the deacetylases are released during hepatocyte priming remains obscure and may involve other factors, such as NF- $\kappa$ B, a key factor in the activation of *c-myc* and other immediate-early genes during liver regeneration [44]. On the other hand, the persistence of pocket proteins does not mean that they are ineffective in the priming process. For example, in the growth arrest during macrophage terminal differentiation, *i.e.*, in the exit from cell cycle to enter G<sub>0</sub>, Ets factors are replaced in the *c-myc* promoter by the repressor METS, which, in turn, recruits a Sin3A-deacetylase-corepressor complex and the interplay between METS and E2F-pRb complexes can provide a mechanism to exit from the cell cycle [45]. The mechanisms for interaction between METS and E2F-pRb are not known, but *Id2* protein might be involved in them provided its capability to bind pocket proteins as well as repressor complexes. Obviously, more research is

needed to ascertain the existence of any of these mechanisms. The use of knock-out mice lacking *Id2*, in which some aspects of the mechanisms of lactation have been studied [46], may be of great interest to clear up the exact role of *Id2* in *c-myc* control and in hepatocyte priming.

Finally, we wish to emphasize that the occupancy of *c-myc* promoter shows an obvious correlation between cirrhotic liver and the priming phase after PH. Cirrhosis constitutes a risk factor for hepatocellular carcinoma (HCC) [47] although the mechanisms for the cirrhosis–HCC progression are still poorly understood. The expression of *c-myc* in human and animal cirrhotic livers is generally considered to precede the formation of HCC [48-50], so in view of our data it could be hypothesized that cirrhotic hepatocytes are in a certain sense “primed” for proliferation. It should be desirable to further explore this question, as the feasibility of the ChIP assays might made them a useful technique of prognostic value in evaluating the risks of cirrhosis to HCC transition.

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## FIGURE LEGENDS

**Figure 1 Expression of *Id2* and *c-myc* genes during liver regeneration.** The figure gives the steady-state levels of *Id2* (A) and *c-myc* (B) mRNA vs. time after PH, as shown by semi-quantitative and quantitative RT-PCR. In the latter instance, histograms give the data for controls (t = 0) and after PH, corrected relative to the level of 18 S rRNA. The value of control was arbitrarily set to 1. Samples were run in triplicate for two independent series of animals. The bars show the standard deviation. The melting curves are also shown to check that a single specific amplicon is being generated.

**Figure 2 Complex-associated forms of *Id2* in the nuclei of quiescent liver.** Nuclear extracts were immunoprecipitated with antibodies against p130, *Id2*, mSin3A and E2F4. The proteins in the immunoprecipitates were subjected to electrophoresis (8% PAGE for p130 and 15% PAGE for *Id2*) and analyzed by Western blotting using the antibodies against p130 (B, upper panel) and *Id2* (B, lower panel). To assess the specificity of p130 and *Id2* antibodies, the input sample (5% of total nuclear aliquot) was also analyzed by Western blotting (A). As a control, a sample was treated in the immunoprecipitation protocol with a non-related antibody (NR Ab). A further control was carried out in the experiment without added antibody (No Ab).

**Figure 3 *In vivo* binding of several factors to *c-myc* gene at different times after PH.** (A) Analysis of *c-myc* promoter by ChIP assay with E2F4, p130, mSin3A and *Id2* antibodies. For each time a sample of total chromatin (input) was included in the PCR reactions. No Ab: no antibody. (B) PCR products obtained with oligonucleotides specific for  $\alpha$ -actin gene promoter, included as a negative control. (C) RNAPol ChIP analysis of the *c-myc* transcription. The same cross-linked chromatin samples used in (A) and (B) were immunoprecipitated with an anti-RNA polymerase II antibody, and the immunoprecipitates were analyzed by PCR using specific primers from the *c-myc* coding region. The presence of RNA polymerase in these regions is taken as a measure of actual transcription. As negative and positive controls, respectively, the PCR analysis of the immunoprecipitates with primers from the  $\alpha$ - and  $\beta$ -actin gene coding regions are also added.

**Figure 4 Analysis of the *Id2* gene for binding of *c-myc* and RNA polymerase *in vivo* to the promoter and for transcription after PH.** Immunoprecipitation of formaldehyde-cross-linked chromatin was carried out in rat control liver and at several times after PH, by using antibodies

against *c-myc* (A) or RNA polymerase II (B) and (C). Immunoprecipitates were analyzed by PCR with primers specific for the *Id2* promoter (A and B) or for the *Id2* coding region, to show actual transcription of the gene (RNAPol ChIP assay, panel C). In the right panel of (A), the PCR products obtained with oligonucleotides specific for  $\alpha$ -actin gene promoter were included as a negative control.

**Figure 5 Expression of the *Id2* gene in rat liver 28 days after BDL.** (A) RT-PCR analyses of *Id2* mRNA for three representative liver samples obtained 28 days after BDL. (B) RT-PCR analyses of *c-myc* mRNA in the same samples as in A. (C) Western blot analysis of Id2 protein at 28 days after BDL,  $\beta$ -actin was used as loading control. (D) Immunohistochemical localization of Id2 in control rat liver. See Materials and Methods for experimental details. (E) Immunohistochemical localization of Id2 in rat liver 28 days after BDL.

**Figure 6 mSin3A and Id2 are released *in vivo* from *c-myc* promoter in cirrhotic rats when the gene is being transcribed.** The experiment was conducted as for Figure 3, with samples from rat cirrhotic liver at 28 days after BDL. (A) The ChIP analysis was carried out with the antibodies indicated over the PCR results in control and cirrhotic rats. The primers were specific for *c-myc* promoter. (B) Real-time RNAPol ChIP analysis of *c-myc* transcription in normal and cirrhotic rats. An antibody against RNA polymerase II was used for immunoprecipitation of chromatin and the bound DNA was analyzed with primers from the third exon of *c-myc* gene.

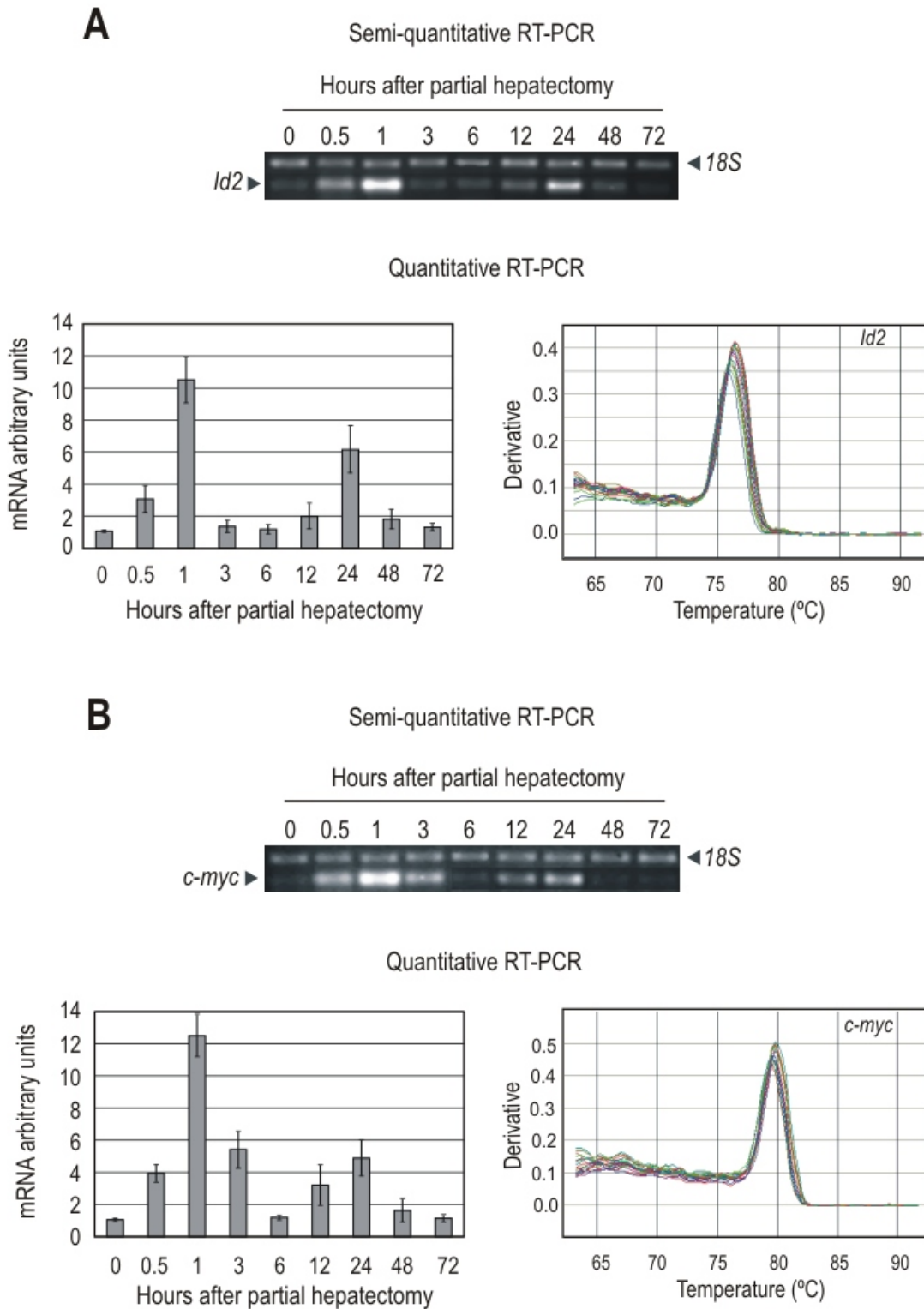


Figure 1

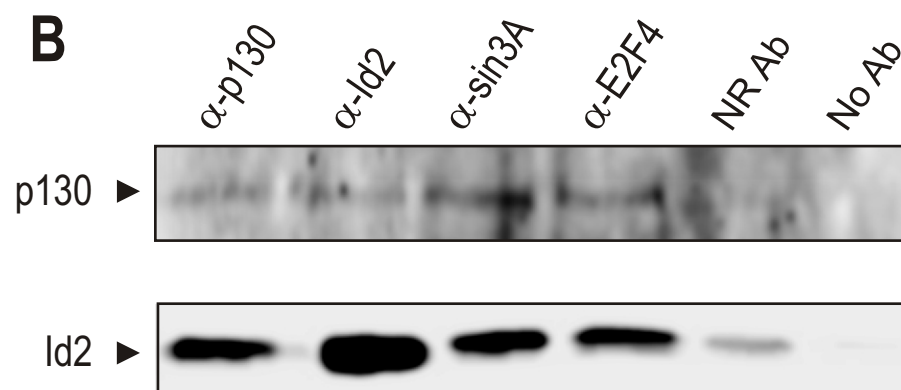
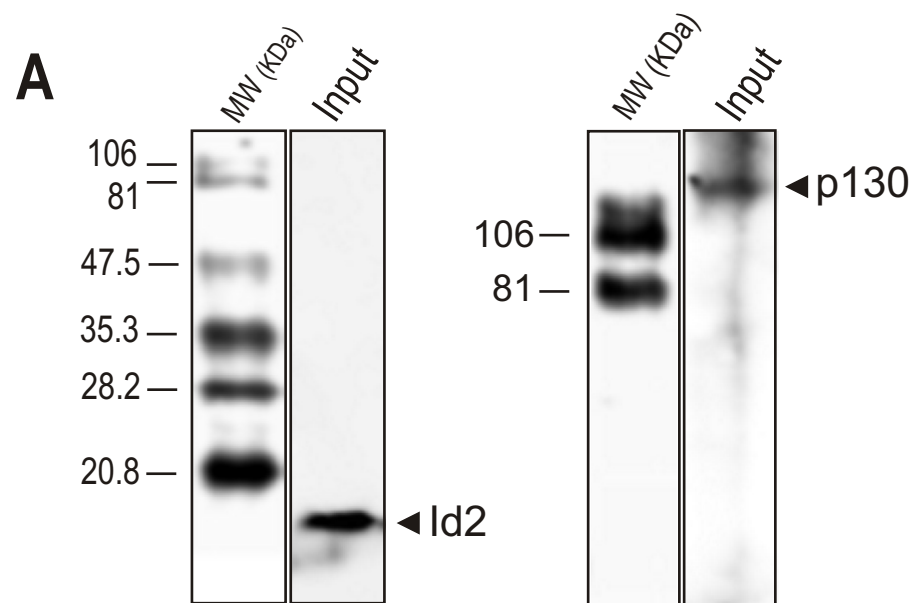


Figure 2

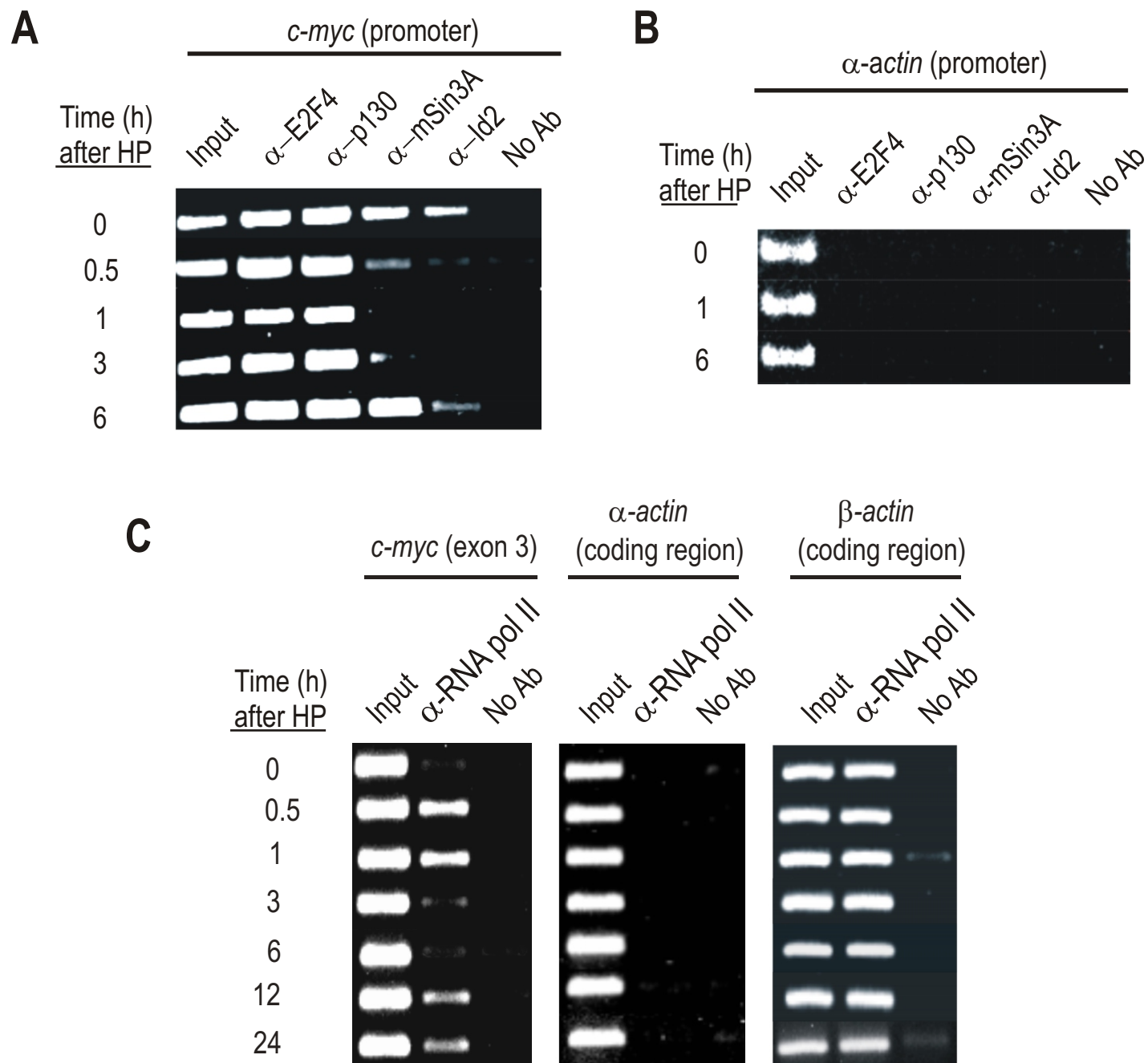


Figure 3

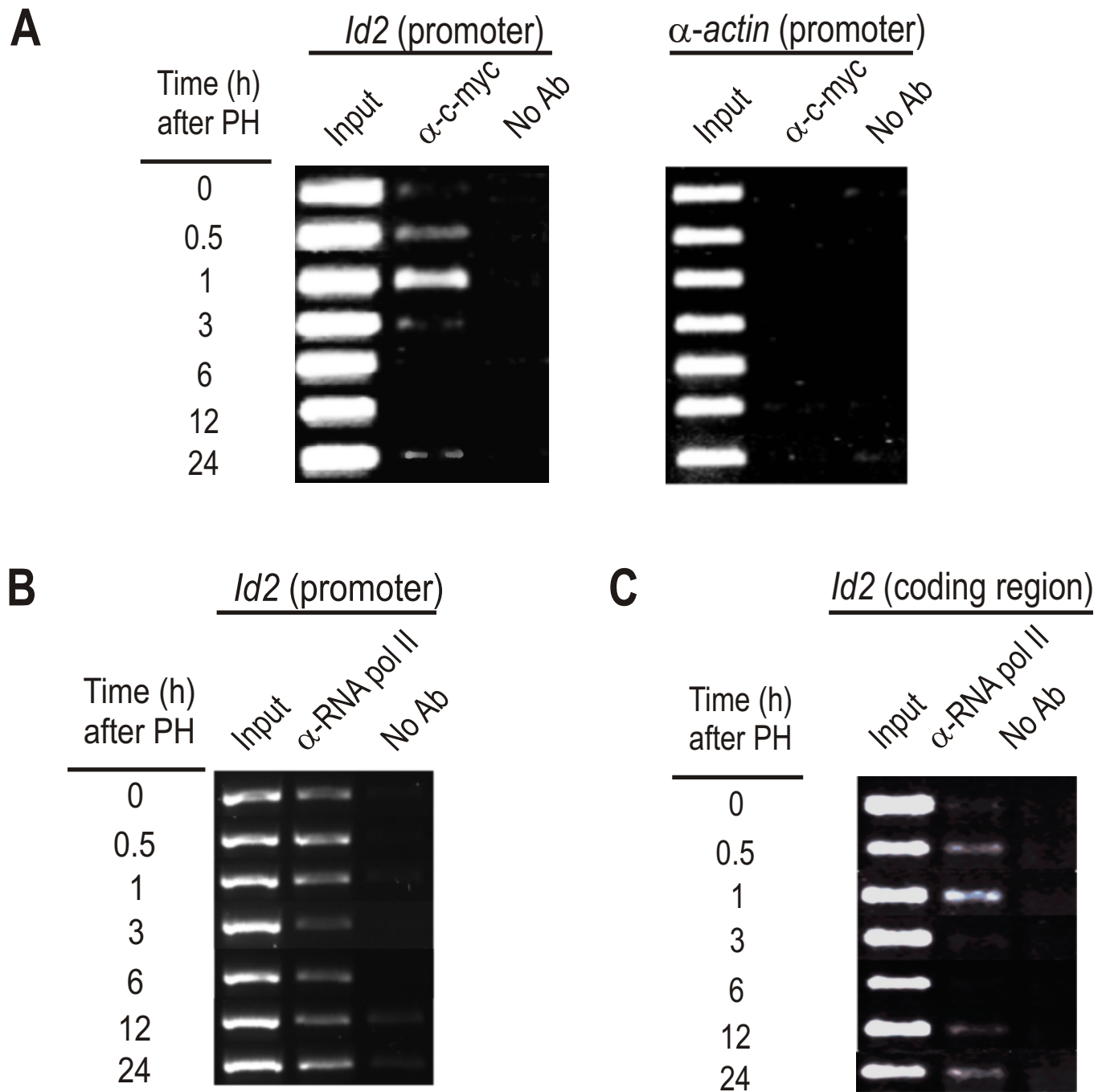


Figure 4

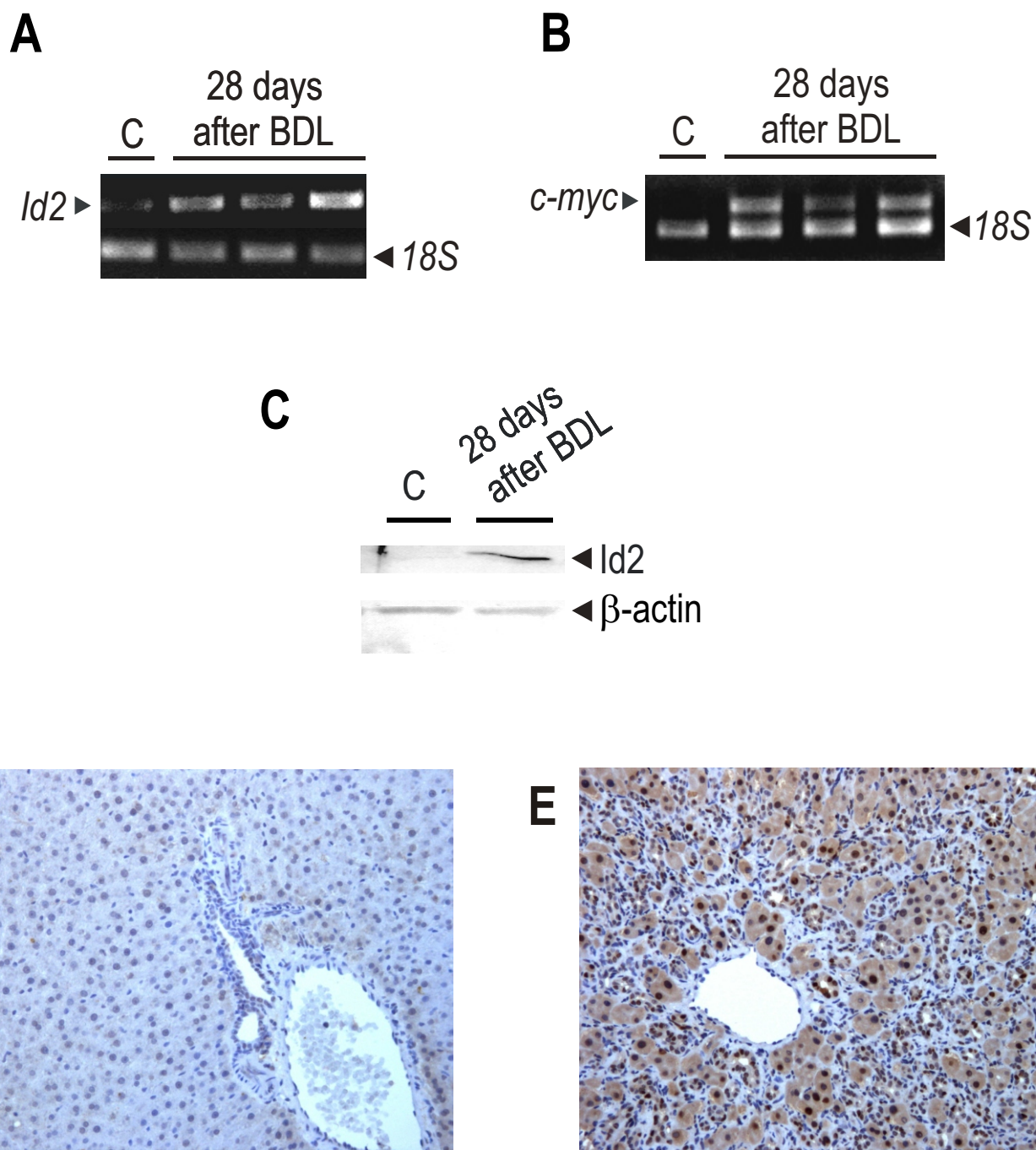


Figure 5

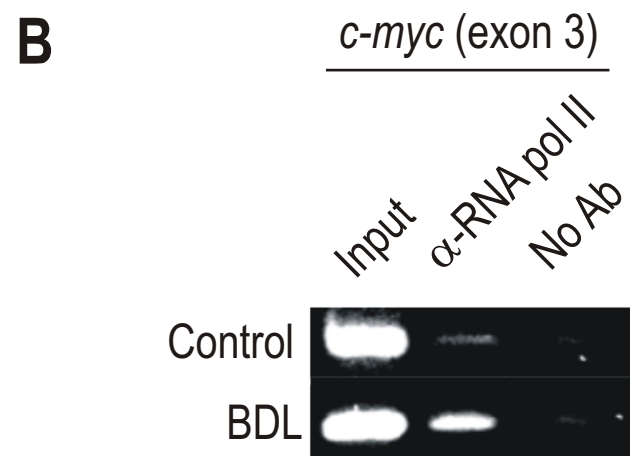
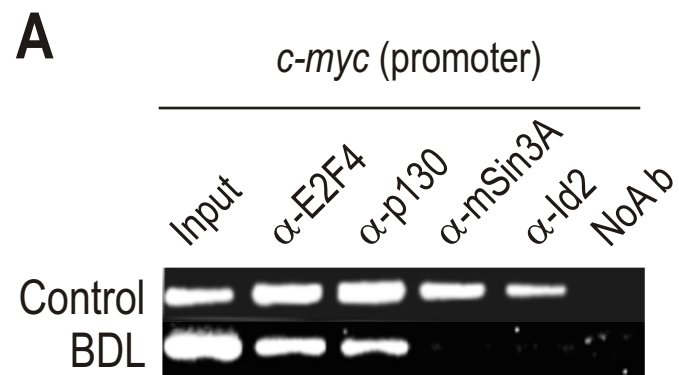


Figure 6