RNAPol-ChIP: a novel application of chromatin immunoprecipitation to the analysis of *real-time* gene transcription

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ABSTRACT

We describe a procedure, RNAPol-ChIP, to measure actual transcriptional rate. It consists of the detection, by chromatin immunoprecipitation (ChIP), of RNA polymerase II within the coding region of genes. To do this, the DNA immunoprecipitated with polymerase antibodies is analysed by PCR, using an amplicon well within the coding region of the desired genes to avoid interferences with polymerase paused at the promoter. To validate RNAPol-ChIP, we compare our results to those obtained by classical methods in several genes induced during either liver regeneration or acute pancreatitis. When short half-life mRNA genes are studied (e.g. *c-fos* and *egr1*), RNAPol-ChIP gives results similar to those of other procedures. However, in genes whose mRNA is more stable (e.g. the hemopexin, *hpx*, gene) RNAPol-ChIP informs on real-time transcription with results comparable to those of methods such as nuclear run-on or run-off, which require the isolation of highly purified nuclei. Moreover, RNAPol-ChIP advantageously compares with methods based on the analysis of steady-state mRNA (northern blot or RT–PCR). Additional advantages of RNAPol-ChIP, such as the possibility of combining it with classical ChIP analysis to study transcription-associated changes in chromatin are discussed.

INTRODUCTION

The study of the regulation of gene expression in eukaryotes is now one of the topics to which enormous personal and economic efforts are being dedicated. In view of the increasing evidence that chromatin structure plays a decisive role in regulating gene expression, there is an increasing tendency to simultaneously study both structural and functional aspects of gene regulation. As an illustrative fact, it may be mentioned that more than 400 review articles have been dedicated to this issue in the top journals in the last 5 years.

The studies on the regulation of gene expression require the quantification of transcriptional rate, which usually involves the detection of specific mRNAs. The methods commonly used for this purpose are northern blotting and *in situ* hybridization (1), RNA protection assay (2,3), semi-quantitative and quantitative RT–PCR (4–6) and nuclear run-on/off (7,8). The pros and cons of all these techniques have been discussed in many reviews [see, for instance, ref. (9)].

Northern blotting, because of its relative ease, is widely used. However, it presents some limitations resulting from the difficulty to control the efficiency of RNA transfer and blotting to membranes, as well as from ill-defined factors that affect the probe hybridization to nucleic acids on a solid support. *In situ* hybridization is based on the same principle as northern blot and it is the sole technique that allows location of transcripts to specific cells within a tissue. These two techniques are not sensitive enough to detect low-level gene expression and they are not accurate enough to quantify the full range of gene expression.

RNase protection assays involve the hybridization of a labelled probe to the target mRNA. Its sensitivity is at least 10-fold higher than northern blot, thus allowing the low-abundant mRNAs to be detected (10,11). However, the procedure is time consuming as it has to be optimized for each target mRNA.

RT–PCR-based assay is now, due to its unparalleled sensitivity, the technique of choice to quantify low-abundance mRNAs and to detect it in a small number of cells (9,11). Several variables that influence amplification, such as

PCR cycling conditions, concentration of the reactives or oligonucleotide composition (12), must be determined and controlled. Moreover, due to the sensitivity of RT–PCR, very small amounts of genomic DNA contamination in an RNA preparation may serve as a template for amplification and produce artefactual results.

In all of the above-mentioned techniques, only the steady-state levels of the mRNA are determined, as pointed out by Bustin (5). In mammalian cells, there are different mechanisms leading either to selective mRNA stabilization or to enhanced degradation (13), which result in the half-life of a given mRNA to range from 20 min to 24 h. It has been found in a recent analysis of the mRNA level in stress-regulated genes that changes in transcription affect 47% of the genes, while the stability of mRNA has an effect in ∼53% of them (14). To avoid these difficulties, run-on or nuclear run-off transcription assays are often used to study transcription at real time. These approaches allow measuring the transcriptional activity of a given gene in its genuine structural and regulatory cellular context (7,8). However, serious problems in the interpretation of results can arise from the artificial activation of paused RNA polymerase during transcriptional reaction. Eick *et al.* (15), using the human proto-oncogene c-*myc* as an example, showed that activation of paused, elongation-incompetent polymerases produces a strong signal in cells that do not express c-*myc*, due to the artefactual transcription of the first exon. On the other hand, nuclear run-on requires the isolation of purified nuclei, which may represent an additional difficulty in some cells or tissues.

Chromatin immunoprecipitation (ChIP) is a powerful approach that allows the analysis of the interaction of transcriptional factors and chromatin–regulator complexes with DNA in living cells, thereby providing a *live cell picture* of the native chromatin structure and factors bound to genes in different functional states [see, for instance, ref. (16)]. Typically, ChIP methodology first involves the protein–protein and protein–DNA crosslinking. Isolated, crude chromatin is then sonicated to yield small fragments, normally of 300–1000 bp of average size. Antibodies against a protein that supposedly binds to a given tract of DNA are then used to immunoprecipitate fragments of chromatin. PCR analysis of the immunoprecipitate, using oligonucleotides spanning the desired DNA sequence, reveals whether that protein is actually bound *in vivo* to that DNA region.

We describe in this work a novel application of the ChIP methodology, which we have called RNAPol-ChIP, consisting of the detection of RNA polymerase II within the coding region of the genes. The procedure, simpler than nuclear run-on or run-off, allows the quantification of actual transcriptional rates of target genes and circumvents the disadvantages of methods measuring just the steady-state mRNA level. We report the results obtained with RNAPol-ChIP in the analysis of the transcription of several genes (c-*fos*, *egr1* and *hpx*, the hemopexin gene) in different experimental models. While our results are similar to those obtained by classical methods in genes with short half-life mRNA, such as c-*fos* and *egr1*, the results with *hpx*, whose mRNA has a very long half-life, reflect the actual transcriptional rate and its results are markedly different from those obtained with methods based on the detection of steady-state mRNA levels.

MATERIALS AND METHODS

Biological materials

Male Wistar rats of ∼250 g were used in the experiments of liver regeneration after partial hepatectomy (PH) and in the acute pancreatitis induced by taurocholate. Rats were cared and handled according to the 'Current Protocols for Protection of Animals Used for Experimental and Other Scientific Purposes' (Council Directive 86/609/EEC).

Crosslinked chromatin preparation

Liver regeneration was induced by 2/3 PH, as described by Higgings and Anderson (17), under intraperitoneal anaesthesia with ketamine (100 mg/kg body weight) and acepromazine (2.5 mg/kg body weight). Rats were sacrificed at the indicated times after PH. Livers were excised and treated with 1% formaldehyde for 12 min to crosslink the chromatin and the reaction was stopped by adding glycine to a final concentration of 0.125 M. The liver tissue was disaggregated with a Dounce homogenizer, passed through a 200 μm pore filter and centrifuged at 1500 g for 5 min. The cell pellet was resuspended in cell lysis buffer (85 mM KCl, 0.5% NP40, 5 mM HEPES pH 8.0) supplemented with a protease inhibitor cocktail, incubated on ice for 15 min and centrifuged at 3500 g for 5 min to pellet the nuclei. The pellet was resuspended in nuclear lysis buffer (10 mM EDTA, 1% SDS, 50 mM Tris–HCl, pH 8.1) at a ratio 1:1 (v/w) relative to the initial tissue weight, incubated on ice for 10 min, aliquoted in 1 ml fractions and stored at −80 °C until use for RNApol-ChIP.

Acute pancreatitis was induced by retrograde infusion of 3.5% sodium taurocholate (Sigma) into the biliopancreatic duct as described by Aho *et al.* (18). The correct induction of the acute pancreatitis was confirmed by anatomopathological studies of the pancreas and by measurement of lipase and amylase activities in blood serum. The pancreases from sham-operated rats and from animals after 0, 1 and 6 h of taurocholate treatment were fixed in 1% formaldehyde for 10 min. The crosslinking reaction was stopped with 0.125 M glycine (final concentration) and the samples were processed as describe before for liver.

RNApol-ChIP procedure

Crosslinked chromatin (1 ml of each sample) was sonicated on ice with 7 (liver) or 5 (pancreas) pulses of 10 s and 40% amplitude in a Vibra-Cell VCX-500 sonicator (Sonics and Materials). The average chromatin size of the fragments obtained was ∼500 bp. The sonified chromatin was centrifuged at 14,000 g for 10 min and the supernatants, containing soluble chromatin fragments, were diluted 10-fold with dilution buffer (165 mM NaCl, 0.01% SDS, 1.1% Triton X, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0) supplemented with protease inhibitor cocktail (Sigma). The diluted chromatin fractions were precleared by adding 30 μl/ml of protein A/G agarose (Amersham Biosciences) (previously blocked during 1 h with 100 μg/ml λ-DNA, 500 μg/ml tRNA, 1 mg/ml BSA) and kept for 4 h at 4°C on a rotating plate. The suspensions were then centrifuged at 14,000 g for 30 s to discard non-specifically-bound chromatin fragments. Aliquots from the supernatant (equivalent to 50 μg DNA) were taken, incubated with 2 μg of RNA pol II antibody (Santa Cruz, sc-899) and

left to stay overnight at 4°C under rotation. The samples were then incubated with 50 µl of blocked protein A/G agarose under rotation for an additional period of 4 h. The immuno-complex, containing chromatin fragments/α-RNA pol II/pro-tein A/G agarose, was recovered by centrifugation at 14,000 *g* for 30 s, washed twice with low-salt buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1 mM EDTA, 50 mM Tris–HCl), twice with high-salt buffer (500 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1 mM EDTA, 50 mM Tris–HCl), twice with LiCl buffer (250 mM LiCl, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1 mM EDTA, 50 mM Tris–HCl) and finally twice with TE buffer (0.25 mM EDTA, 10 mM Tris–HCl). During each washing, the suspension was kept under rotation for 5 min at 4°C. An aliquot of the crosslinked chromatin was treated as above, but in the absence of the antibody (*NoAb* fraction) and the first supernatant, after the preclearing with protein A/G agarose, was saved as *Input* fraction. The immu-noselected chromatin was eluted from the protein A/G agar-ose in two consecutive steps by adding 100 µl of elution buffer (1% SDS, 100 mM NaHSO$_3$) each, with 30 s of vigorous vortexing. Both supernatants were combined (immunoprecipitated, *IP* fraction) and incubated at 65°C overnight to reverse formaldehyde crosslinks. The DNA from all these samples was purified with a PCR purification kit (Qiagen) and used for semi-quantitative or quantitative PCR analysis of the target genes. We routinely tested the specificity of chromatin immunoprecipitation by checking the presence of RNA polymerase II in the *IP* fraction by western blotting analysis, carried out with the antibody used to immu-noprecipitate the chromatin fragments. In some instances, we checked by western blot analysis that the RNA polymerase phosphorylated at serine 2 of the C-terminal domain (CTD), an elongation-specific isoform of the enzyme, is also present in the immunoprecipitates prepared for RNApol-ChIP. To do this, an antibody directed against this modified isoform (H5 MMS-129R, Covance) was used. In some cases, RNApol-ChIP was carried out by direct, selective immunoprecipitation of the elongating polymerase, by using the latter antibody.

PCR analysis of immunoprecipitated chromatin

Input, IP and *NoAb* fractions were analysed by PCR with the appropriated primer pairs to amplify products of 180–300 bp in length, corresponding to either the promoter or coding regions of the target genes. For the analysis 1:5000 dilutions of the *Input* and 1:30 of the *IP* and *NoAb* fractions were used. Primer sequences were as follows: c-*fos* promoter, forward 5′-GGGAAAGCGCTCCCGAATGC-3′and reverse 5′-GCA-CCCTCAGAGTTGGTGC-3′ (286 bp product); c-*fos* coding region, forward 5′-TGGACTTGACTGGGGGTCTG-3′ and reverse 5′-CAGGTCCACATCTGGCACAG-3′ (221 bp product, fragment within exon 3); β-*actin* promoter, forward 5′-TTCTACGTTTCCATCCAAGCCGT-3′and reverse 5′-TT-TCTTGTTCGAAGTCCAAGTCCAAGG-3′ (223 bp prod-uct); β-*actin* coding region, forward 5′-AGAGCAAGA GAGGCATCCTG-3′and reverse 5′-GGGTCATCTTTTCA-CGGTTGG-3′ (186 bp product, fragment within exon 2); α-*actin* promoter, forward 5′-AGGATTCCTACGTGGGC-GAC-3′and reverse 5′-TAGAGAGACAGCACCGCCTG-3′ (200 bp product); *hpx* coding region, forward 5′-GGCTTTG-

GTGGCTGGACCTG-3′ and reverse 5′-ACTTTCTGGGGC-TGAGGCAG-3′ (226 bp product, fragment within exon 10); *egr1* coding region, forward 5′-CAGAAGCCCTTC-CAGTGTCG-3′ and reverse 5′-GATGGGTAGGAGGTAGC-CAC-3′ (263 bp product, fragment within exon 2).

PCR fragments were size-fractionated by 2% agarose gel electrophoresis, stained with ethidium bromide and analysed with a FLA3000 electronic autoradiography system (Fujifilm) using Image Gauge V3.12 software.

RNA isolation and analysis of nucleic acid level by RT–PCR and quantitative PCR

Total RNA was isolated by the guanidinium thyocianate method (19). In the case of the pancreas, the tissue was pre-viously immersed in 1 ml of RNA-later solution (Ambion) to stabilize the RNA. The isolated RNA (2 µg/lane) was size-fractionated by electrophoresis in a 1% agarose/formalin gel and stained with ethidium bromide to assess the quality of the RNA. The cDNA used as template for amplification in the PCR assay was constructed by reverse transcription reaction using SuperScript II (Invitrogen), with random hexamers as primers, starting with equal amounts of RNA. As a PCR inter-nal control, 18S rRNA was simultaneously amplified. To obtain similar PCR band intensities competitor oligonucleo-tides were added to the assay in a proportion 3/7 (normal 18S rRNA oligonucleotides/competitor 18S rRNA oligonucleo-tides). The competitors correspond to the same 18S rRNA oligonucleotide sequence, except that their 3′ termini were blocked with an amino group.

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

RESULTS

Principle of the technique

The RNApol-ChIP approach for transcription analysis consists of the detection of the RNApol II presence in the coding region of target genes. The method uses the ChIP technique with an antibody against RNApol II. Since the average size of sonic-ated chromatin is ∼500 bp (Figures 1A and 3A), the PCR analysis of the immunoprecipitate should be performed by using oligonucleotides spanning a region far enough within the coding region (>1000 bp from the start of transcription) to avoid immunoselection of non-elongating, paused RNApol II at the promoter of the target genes.

Experimental validation of RNAPol-ChIP assay

Gene transcription during liver regeneration. To validate the RNApol-ChIP assay for the analysis of gene transcription, we studied several genes of known expression pattern, and com-pared the results with those obtained with some of the classical methods described in the literature. Figure 1 shows the results of c-*fos* gene transcription analysis in liver tissues after PH.
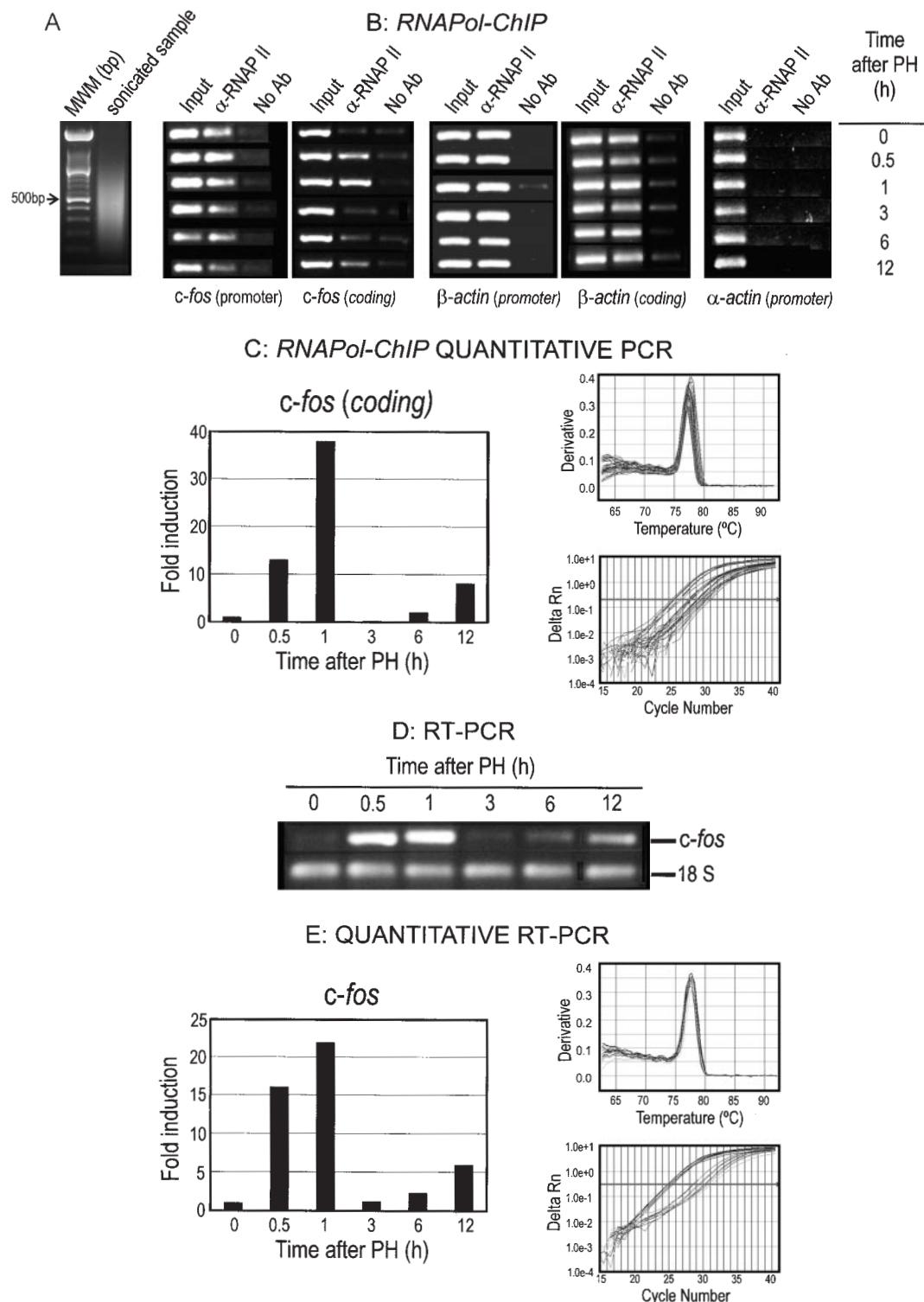
Figure 1. Time-course of c-*fos* transcription in rat liver after PH. (**A**) RNApol-ChIP analysis of c-*fos* transcription. The panels in the top row show the results of the PCR analysis of DNA extracted from input samples (*I*), the immunoprecipitates with anti-RNA polymerase II antibody (*IP*) and samples treated without antibody (*NA*) at several times after PH, using amplicons (see the text for details) from the promoter or coding regions of the indicated genes. The left panel shows an agarose gel electrophoresis of the sonicated samples to estimate the size of chromatin fragments. The lower panels show the results of a real-time quantitative PCR. To do this, DNA samples obtained in the assay (*input*, α-RNA pol II *IP* and *No Ab*) were used. The results of the *No Ab* samples at each time point were subtracted from those of the IP samples, the resulting figures divided by their corresponding input and finally plotted in a scale in which the final value of the control (0 h after PH) was arbitrarily set to 1. The melting curves of the amplicons and a plot of amplicon production versus number of cycles are given as PCR product verification. (**B**) RT–PCR analysis of c-*fos* transcription. The agarose gel electrophoresis of the RT–PCR products of c-*fos* and 18S *rRNA*, used as control, is shown for several times after PH. Quantitative RT–PCR analysis for c-*fos* is given below. The c-*fos* data were divided by those of the control 18S *rRNA*, and the histogram were constructed as above. The verification of PCR products was shown as in (A).

Under these conditions, c-*fos* is expressed as an immediate-early gene [reviewed in ref. (20)]. The RNApol-ChIP analysis was carried out by measuring the binding of the RNA pol II to the coding region of the gene. The measurement was done by PCR, using an amplicon within the exon 3 or, more accurately, by quantitative PCR within the same region (Figure 1A). In accordance with the data of other authors, the expression of c-*fos*, as measured by the RNApol-ChIP assay in the coding region, increases immediately after PH and passes a maximum at 1 h, which is followed by a decrease until 3 h after PH. A second, but smaller increase, was observed later after PH (Figure 1A). At this point, it has to be taken into account that the half-life of c-*fos* mRNA is very short. For instance, the early data of Rahmsdorf *et al.* (21) showed that this half-life in human fibroblasts is 9 min. This fact explains why the data obtained by RNApol-ChIP substantially agree with those of RT–PCR (Figure 1B). Interestingly, RNA polymerase II is continuously bound to the c-*fos* promoter (Figure 1A). This result is in accordance with the idea that the gene is a 'potentially active gene', in which RNA pol II is paused at the promoter, waiting for the correct cellular signal to start transcription. As mentioned above, this may be a cause of artefacts when nuclear run-on is used to determine the transcriptional rate (15). Similar results (unpublished data) were obtained in the analysis of c-*myc*, another immediate-early gene in liver regeneration (20) with short half-life mRNA.

When the gene of β-actin, which is constitutively transcribed during liver regeneration, was analysed with RNApol-ChIP, a permanent binding of the enzyme was observed both in the promoter and in the coding region of the gene (Figure 1A), in contrast to the behaviour of c-*fos*. The analysis of α-actin gene as a negative control is also shown in Figure 1A.

Contrary to the above results, when genes whose mRNA is more stable are studied, RNApol-ChIP eludes the inconveniences of northern blot analysis and RT–PCR and gives results similar to those obtained by nuclear run-on/off. This is the case of *hpx* gene, whose half-life is significantly greater than 12 h (22). Figure 2A shows that RNA pol II, which is absent from the coding region in the liver of control animals, appeared bound between 3 and 12 h after PH, and it returns to the basal level 24 h post-PH (Figure 2A). When the mRNA level was measured by RT–PCR (Figure 2B), an increase over the basal concentration is also observed 3 h after PH. However, the maximum occurs at 24 h, and the level is still relatively high 48 h after PH. The above data, obtained by RT–PCR, are identical to those found by Albrecht *et al.* (22) by northern blot. These results clearly show that the measurement of the steady state level of mRNA does not correlate with the *actual* transcriptional process. For instance, 24 h after PH, when the maximum level of mRNA is reached (Figure 2B), transcription has already ceased, as revealed by the absence of RNA pol II from the coding region of the gene (Figure 2A).

Gene transcription during acute pancreatitis. We next studied the applicability of RNApol-ChIP to other experimental systems. The induction of acute pancreatitis results in the immediate-early induction of several genes, such as c-*fos* and *egr1* (23). Pancreas is an adequate biological material for this study, because its elevated RNase activity may make the isolation of mRNA, a mandatory step for
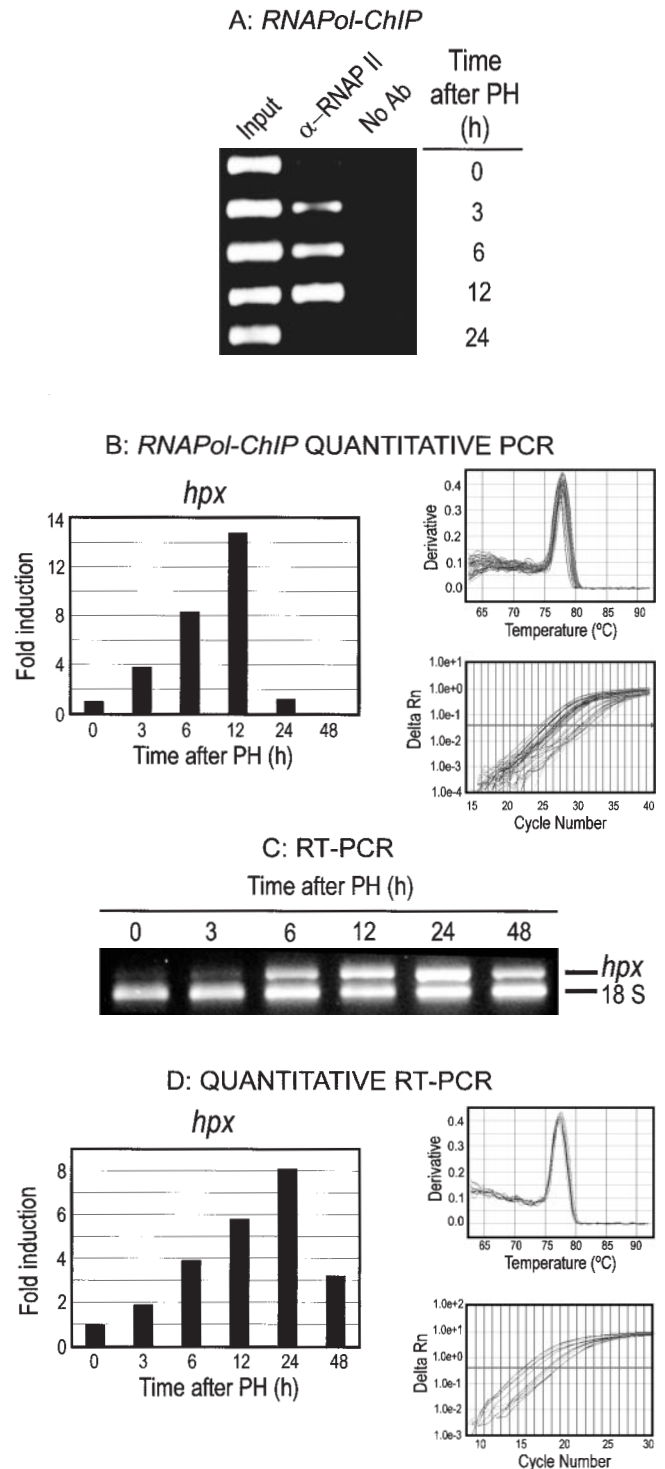

Figure 2. Transcriptional analysis of *hpx* in rat liver after PH. (**A**) RNApol-ChIP assay of *hpx* gene transcription. The data given are as in Figure 1A, except that they correspond to an amplicon from the coding region of the *hpx* gene. (**B**) RT–PCR analysis of *hpx* transcription. The experimental protocol was similar to that described in Figure 1B.

RT–PCR analysis, highly problematic. To avoid degradation, a previous stabilization of RNA in the tissue with RNA later is required for RT–PCR analysis, as described under Materials and Methods. Both RT–PCR and quantitative RT–PCR
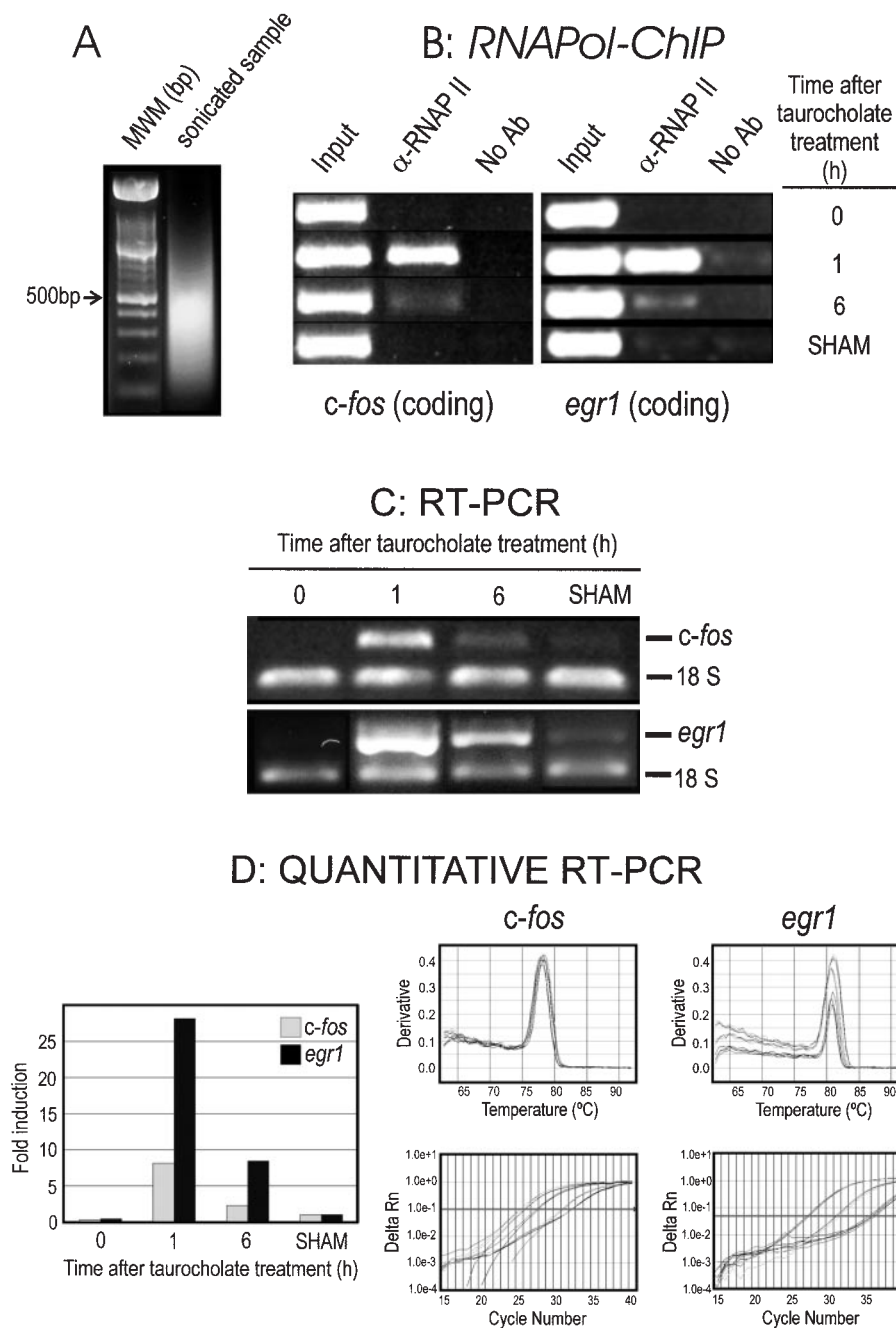
Figure 3. Transcriptional analysis of c-*fos* and *erg1* in rat acute pancreatitis induced *in vivo* by taurocholate. (**A**) RNApol-ChIP assay of c-*fos* and *erg1* transcription. The results of semi-quantitative PCR analysis of DNA, using amplicons from the coding region of c-*fos* and *erg1* genes (see the text for details), were shown for several times after the infusion of taurocholate into the biliopancreatic duct, and in a sham-operated rat (SHAM). The left panel shows an agarose gel electrophoresis of the sonicated samples to estimate the size of chromatin fragments. (**B**) RT–PCR analysis of c-*fos* and *erg1* transcription. The data were obtained and plotted as in Figure 1B.

(Figure 3B) analysis starting with stabilized samples show that c-*fos* and *egr1* mRNAs appeared 1 h after the onset of pancreatitis to rapidly decrease at later times, and that their presence is almost negligible after 6 h. RNApol-ChIP gave identical results (Figure 3A), with the difference that no RNA stabilization step is required. The behaviour of c-*fos* gene in pancreas differs from that observed in regenerating liver in that the RNA polymerase is not paused at the promoter of the inactive gene (results not shown).

We have also carried out an RNApol-ChIP of the pancreatitis samples with an antibody directed against the elongation-competent RNA polymerase isoform, i.e. phosphorylated at the serine 2 of CTD. The semi-quantitative PCR analysis of the *IP* samples for amplicons from the c-*fos* gene gave results similar to those of Figure 3A (data not shown). As expected, no signal was obtained from the promoter because phosphorylation in CTD serine 2 only occurs after the polymerase has started transcription [reviewed in ref. (24)].

DISCUSSION

We describe in this paper a novel application of the ChIP methodology to the study of gene transcription in real time. This procedure uses the presence of RNA pol II within the coding region of the target genes as a way to measure transcription. The selected region has to be far away the transcription initiation site (>1000 bp) to avoid the selection of chromatin fragments in which the RNA pol II may be paused at the promoter of the gene. Of course, this is not the first time that ChIP analysis was used to detect the presence of RNA polymerase in the coding regions of some genes, but the aim of the previous reports was markedly different from our purpose. For instance, the ChIP technique has been used to study the role of the chromatin-remodelling yeast ATPase Isw1p in the elongation process (25) and also to map the changes in CTD phosphorylation along the genes (26,27) but, to the best of our belief, the detection of RNA polymerase within the coding region of genes as a method to quantify transcription constitutes a novel way of using the ChIP assay.

We have validated the RNApol-ChIP assay by comparing its results with those obtained by classical methods in two rat experimental systems that affect the level of gene expression: liver regeneration after PH, and acute pancreatitis induced by taurocholate. PH, stimulates liver cells to proliferate and generates an extensive sequential process of gene expression (20,28–30). Some of the immediate-early or primary response genes, such as c-*fos*, c-*myc*, *jun*B and *jun*D, exhibit a very rapid and significant enhancement of their mRNA levels when cells are induced to proliferate. The induction of acute pancreatitis either by caerulein or taurocholate also results in the up-regulation of several genes, as recently demonstrated by Ji *et al.* (23) using DNA microarrays. The level of c-*fos* mRNA increases three to four times after 1 h of pancreatitis depending on the method of inducing it. The *egr1* gene, which codes for a transcriptional factor playing a pivotal role in regulating inflammatory parameters (31), is also immediately up-regulated after the onset of pancreatitis (23). All these factors led us to select c-*fos* and *egr1* as the target genes of our study in both, liver regeneration and acute pancreatitis. As a model of gene with a long mRNA half-life (>12 h), we selected the hemopexin gene (*hpx*), which is also induced in liver regeneration, albeit at a later time (22).

When short-life mRNA genes (e.g. c-*fos* and *erg1*) were analysed, the results obtained by RNApol-ChIP and by RT–PCR were identical. The induction of *erg1* occurs shortly after the onset of acute pancreatitis and reaches a maximum at 3 h, to decline to basal values at 6 h (Figure 3). The induction of c-*fos* gene in acute pancreatitis follows a similar pattern, which is in turn almost identical to that shown by this gene in liver regeneration (Figure 1). In all these experiments, quantitative RT–PCR and quantitative PCR analysis of the RNApol-ChIP data gave almost identical results. However, when the transcription of *hpx* was studied, RNApol-ChIP gave results markedly different from those obtained by RT–PCR (Figure 2). Our results are identical to those found by Albrecht *et al.* (22) using nuclear run-off analysis (compare Figure 2A with Figure 2 of the Albrecht *et al.* paper).

The precedent paragraphs summarize the first group of advantages of our procedure. RNApol-ChIP can be used, in combination with quantitative PCR, to measure transcriptional rate with accuracy similar to that of other procedures. Moreover, when the stability of gene transcripts results in a long half-life for mRNAs, RNApol-ChIP circumvents the inconveniences of methods based on evaluation of the level of steady-state mRNA, and gives results comparable to those of nuclear run-on or run-off in the measuring of real-time transcription. Nevertheless, the latter techniques require the isolation of highly purified nuclei, which is not necessary in the RNApol-ChIP procedure. The possibility of using RNApol-ChIP in pancreatitis with no need for stabilizing RNA against RNase digestion shows that our method can be easily employed in samples with high RNase content. Some other inconveniences of the classical techniques to evaluate transcriptional rate, which have been mentioned in the introduction, can also be avoided by using RNApol-ChIP. Among them we may emphasize the artefactual activation of paused RNA polymerase during the transcriptional reaction in run-on experiments. It has been mentioned that this may cause troubles in studying the transcription of c-*myc* (15). In our method, however, in spite of the permanent presence of RNA pol II on the promoter, RNApol-ChIP allowed us to separate the signals of the presence of the enzyme on the promoter, a consequence of pausing, and on the coding region, which reflects the actual transcription of the gene (Figure 1A).

Our method possesses the additional advantages that a single preparation of chromatin may be used for immunoprecipitation with many antibodies, providing information on chromatin structure and on the factors bound to it. Moreover, a single immunoprecipitation with an anti-polymerase antibody may afford the study of as many genes as desired simply by using different specific primer pairs for PCR analysis. The coincidence of the results obtained for the coding region of the c-*fos* gene in acute pancreatitis with two antibodies, namely one directed against RNA polymerase and another one against its isoform phosphorylated at serine 2 offers an additional proof of the validity of the method proposed in this paper. At first glance, it would seem preferable to systematically use the second antibody for RNApol-ChIP, but we prefer to immunoprecipitate with the general antibody, because a single immunoprecipitate can be used for the quantification of transcription and for the detection of polymerase paused at the promoter.

Apart from the experiments described here, we have checked the utility of the RNApol-ChIP technique in other experimental systems, such as the stimulation of the MLP29 hepatocyte precursor cell line with phorbol esters (G. Tur and J. L. Rodríguez, unpublished results).

It has not escaped our attention that RNApol-ChIP might be used in combination with DNA microarrays, to analyse the global changes in gene expression induced by different treatments or physiological conditions. Experiments to check this possibility are now being carried out in our laboratory.

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