Volume 250, number 2, 205–210 FEB 07256 July 1989

An in vitro demonstration of peroxisome proliferation and increase in peroxisomal β-oxidation system mRNAs in cultured rat hepatocytes treated with ciprofibrate

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Received 13 March 1989; revised version received 24 April 1989

Using the normal adult rat hepatocytes, plated on rat tail collagen-coated dishes and fed a chemically defined medium, we demonstrate here that ciprofibrate at 0.1 mM concentration, increases significantly the mRNA levels of fatty acyl-CoA oxidase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein, and thiolase (the three enzymes of the β-oxidation system), and causes peroxisome proliferation. Increase in mRNA levels of these genes was evident within 1 h and was maximal 24 h after the addition of ciprofibrate. In hepatocytes cultured in the absence of ciprofibrate, the basal levels of these enzymes were low and further declined with time. Concomitant treatment of hepatocytes with cycloheximide did not inhibit or superinduce the mRNA levels, indicating that this induction may represent a primary (direct) effect of this compound on the expression of these genes and does not apparently involve short-lived repressor protein(s).

Peroxisome proliferation; mRNA; Tissue culture; (Rat hepatocyte)

1. INTRODUCTION

An elucidation of the molecular mechanisms by which carcinogenic peroxisome proliferators regulate gene expression in liver is necessary for the understanding of the biological implications of peroxisome proliferation [1,2]. Using the cDNAs for the peroxisomal β-oxidation enzymes, it has been shown that the mRNA levels of the β-oxidation genes increase 20- to 30-fold in the livers of rats treated with peroxisome proliferators [3–5]. This increase has been attributed to enhanced rates of transcription of the corresponding genes [5]. The mechanism by which structurally diverse peroxisome proliferators affect transcription of particular genes in a tissue-specific manner [2,5] has been postulated to involve binding of these xenobiotics to specific recognition molecules in responsive cell types [2,6], in a manner analogous to the mechanism of action of estrogens [7] and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [8]. In this study we used primary cultures of rat hepatocytes to determine whether or not the peroxisome proliferator-induced increase in β-oxidation system mRNAs is a secondary response influenced by labile protein(s). We demonstrate here that ciprofibrate, a peroxisome proliferator, rapidly induces the accumulation of mRNAs of β-oxidation system genes in cultured rat hepatocytes and that the induction occurs even in the presence of cycloheximide, an inhibitor of protein synthesis, indicating that it is a primary effect of ciprofibrate on liver cells.

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2. MATERIALS AND METHODS

2.1. *Hepatocyte cultures and treatment*

Normal adult male F344 rats (175–200 g) were used for the

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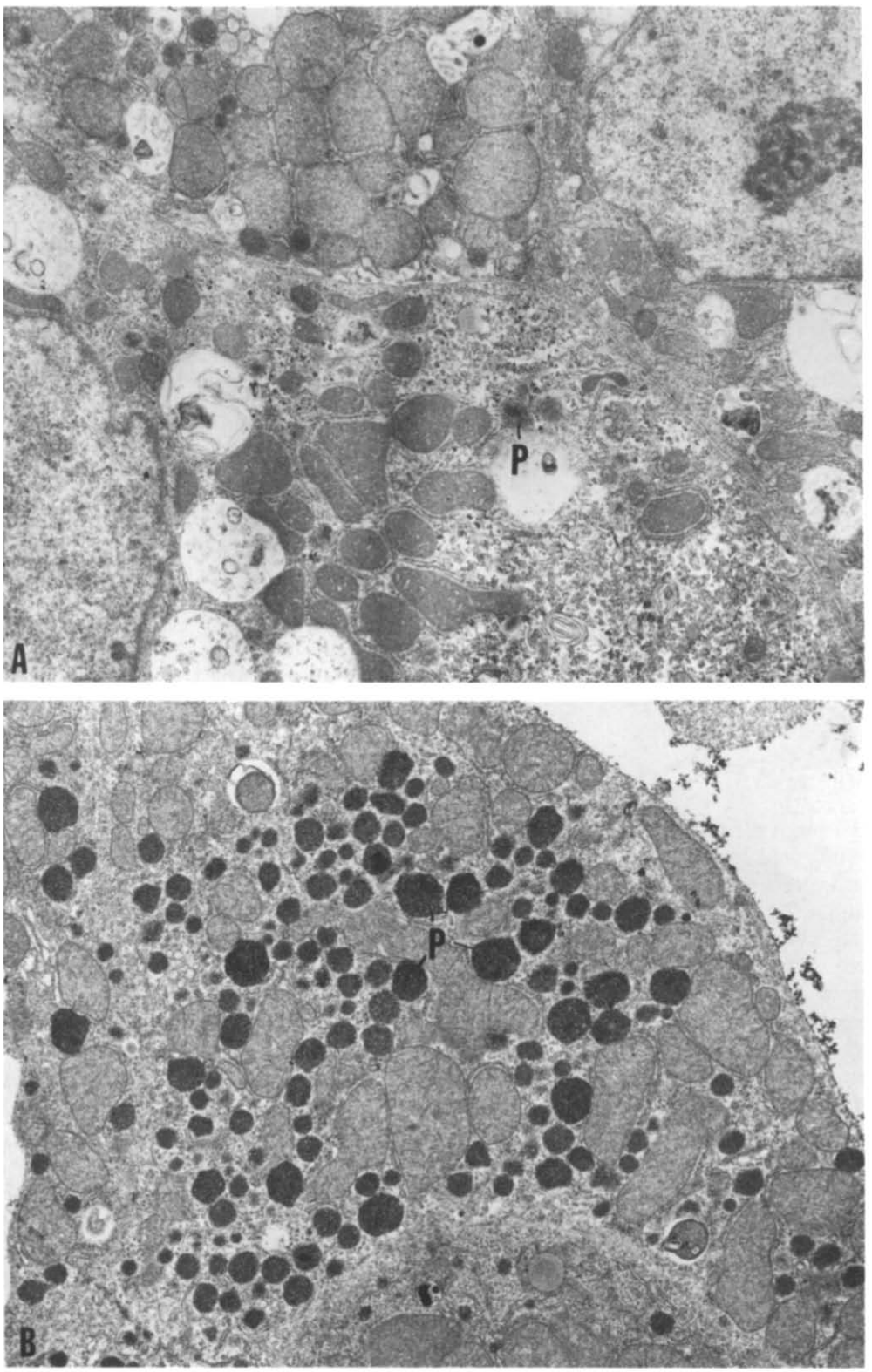
P
A
P
B

Fig.1. Electron microscopic appearance of portions of rat hepatocytes in control (A) cultures and those treated with 0.1 mM ciprofibrate (B) for 5 days. Liver cells in A were processed for routine transmission electron microscopy, whereas the cells in B were processed for the cytochemical localization of catalase by alkaline diaminobenzidine. Increase in the number of peroxisomes (P) is evident in B.

isolation of hepatocytes by in situ collagenase perfusion [9]. The isolated hepatocytes were maintained in primary culture on rat-tail collagen-coated dishes and after attachment fed a chemically defined serum-free medium and 2% DMSO [10]. The cells were maintained in this medium for at least 24–48 h prior to treatment with ciprofibrate and/or cycloheximide. Ciprofibrate was added to the culture medium at 0.05, 0.075, 0.1, 0.5 or 1.0 mM concentrations and the medium changed once every 24 h. The cells were harvested after 2, 3 and 5 days of exposure to the drug and processed for electron microscopy. Selected samples were processed for cytochemical localization of peroxisomal catalase [11] or for immunocytochemical labeling of catalase and PBE by the protein-A gold method [12]. Changes in peroxisome volume and labeling densities were determined as described [12]. For the time dependence of induction of mRNAs of the β-oxidation system, ciprofibrate was added to the culture medium at 0.1 mM concentration and cells were harvested for RNA extraction after 1, 4, 8, 12 and 24 h exposure. To investigate the effect of inhibition of protein synthesis on ciprofibrate inducible β-oxidation mRNAs, hepatocytes were treated for 8 h with ciprofibrate (0.1 mM) in the presence and absence of cycloheximide (10 μg/ml).

2.2. *Isolation of RNA and blot hybridization*

Total cellular RNA from hepatocytes was extracted in guanidinium thiocyanate and analyzed by Northern and dot-blot hybridization as described [13].

3. RESULTS AND DISCUSSION

Dose dependent increases in peroxisome number were observed in hepatocytes treated with ciprofibrate, but higher dose levels (0.5 and

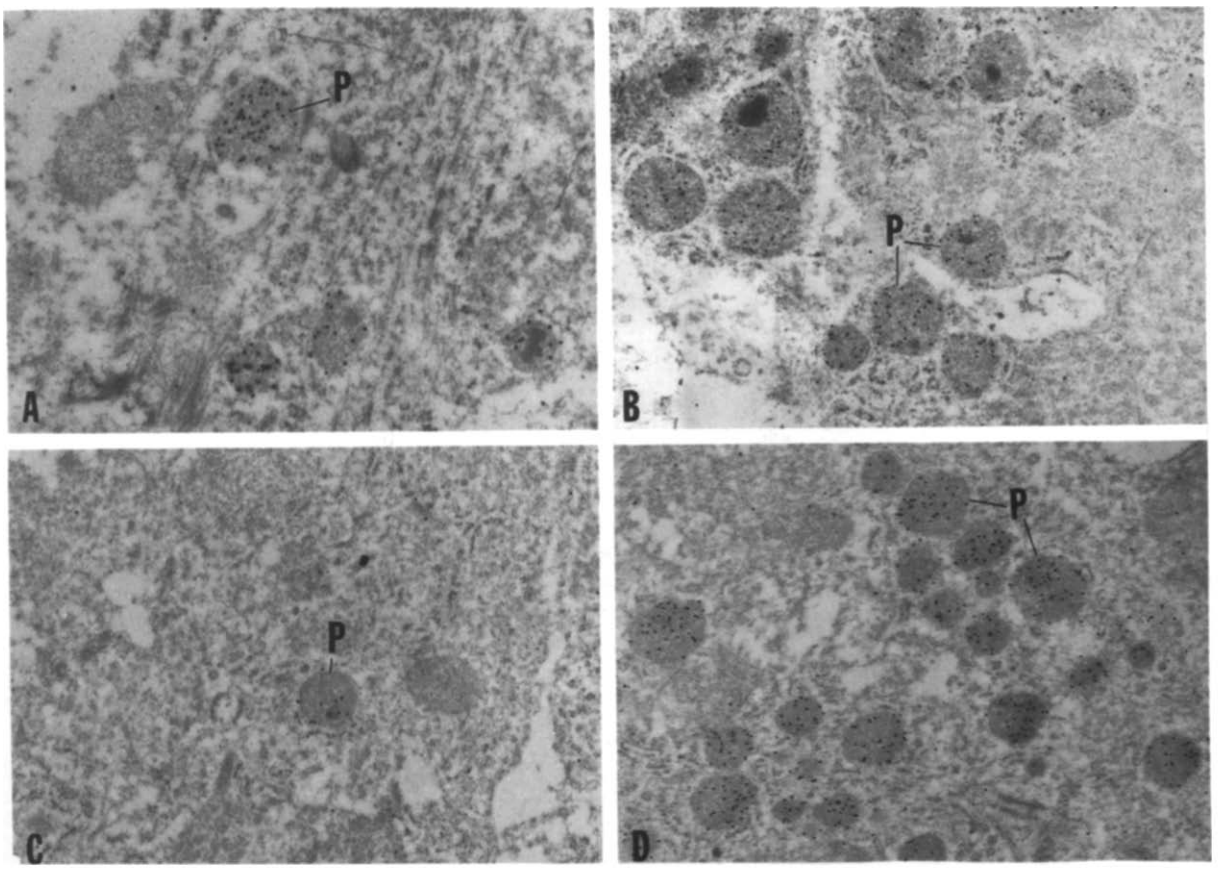

Fig.2. Immunocytochemical localization, by the protein-A gold method, of catalase (A and B) and PBE (C and D) in rat hepatocytes cultured in the absence (A and C) and presence of 0.1 mM ciprofibrate (B and D) for 3 days.

1.0 mM) of this compound appeared cytotoxic. Optimal peroxisome proliferative response was obtained within 3–5 days of treatment with a 0.1 mM concentration of the drug (fig.1). Peroxisomes occupied 9–12% of the cytoplasmic volume in hepatocytes treated for 3 days with 0.1 mM ciprofibrate, whereas in control hepatocytes they accounted for <1% of the cytoplasmic volume. By the protein-A gold immunocytochemical approach, the catalase labeling density in ciprofibrate-treated hepatocytes was 54% of control. In contrast, the labeling density of bifunctional enoyl-CoA hydratase/3-hydroxy acyl-CoA dehydrogenase (PBE) was ~3-fold higher in ciprofibrate-induced peroxisomes when compared to controls (fig.2). The hepatocyte culture conditions described by Isom et al. [10] which were used in this study appear better suited for the purpose of evaluating the peroxisome proliferative property of xenobiotics [14], than those employed previously [15,16].

Northern blot analysis of RNA isolated from cells exposed to 0.1 mM ciprofibrate for 1–24 h revealed that fatty acyl CoA oxidase (FAOxase) (fig.3A), PBE (fig.3B) and thiolase (THL) (not shown) mRNAs were induced by ciprofibrate in a time-dependent manner. The levels of these β-oxidation mRNAs were increased within 4 h and reached a maximum at 12–24 h. By 24 h of ciprofibrate treatment the β-oxidation mRNAs increased 7- to 26-fold compared to controls. In hepatocytes cultured in the absence of ciprofibrate the basal levels of mRNAs of all three β-oxidation genes were low (THL < PBE < FAOXase) and further declined with time in culture. The catalase mRNA increase was <2-fold with ciprofibrate treatment during the 24 h exposure (not shown). No changes in albumin mRNA (fig.3C) levels were noted. Rapid increases in the levels of peroxisomal β-oxidation system mRNAs observed here in primary hepatocyte cultures exposed to ciprofibrate, are consistent with earlier reports of increases in vivo in the livers of rats given a single intragastric dose of structurally different peroxisome proliferators [5,13], and in hepatocytes cultured in the presence of nafenopin [17]. In contrast, Chatterjee et al. [18] noted a 10–15 h delay in the induction of PBE mRNA in rat hepatocytes exposed in vitro to Wy-14643. This discrepancy is most likely due to differences in culture conditions, since Wy-14643 caused a rapid induction in vivo of β-oxidation system mRNAs in livers of rats beginning as early as 30 min after a single intragastric dose [13].

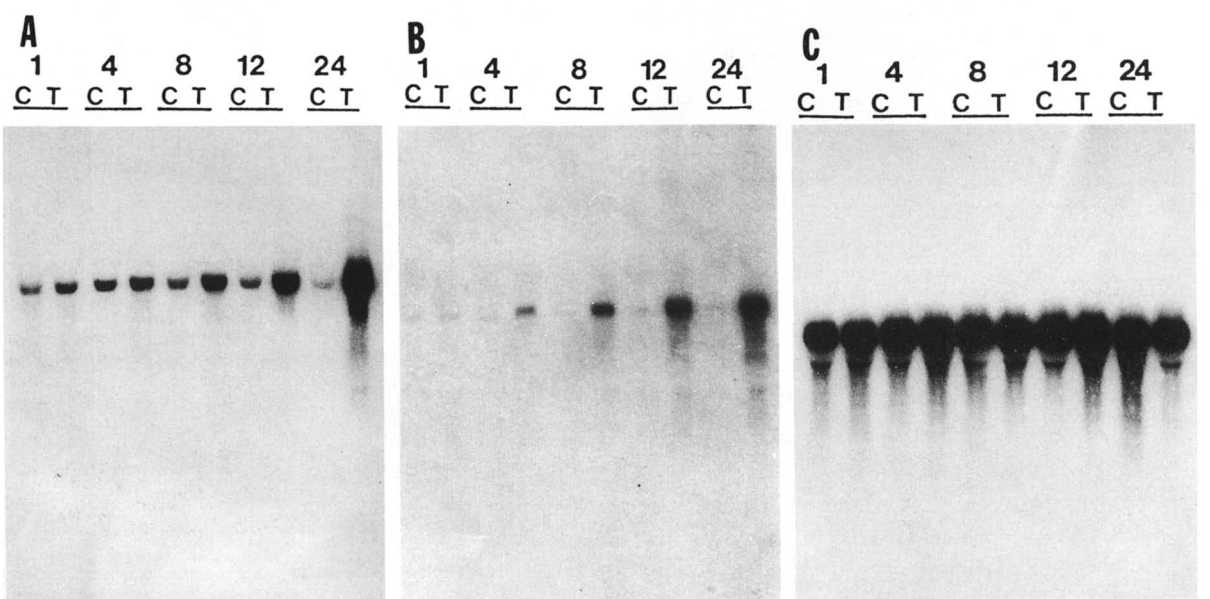

Fig.3. Northern blot analysis (10 μg total RNA/lane) of time-course of changes in the FAOxase (panel A), PBE (panel B) and albumin (panel C) mRNA levels in hepatocytes cultured in the absence (C) or presence (T) of 0.1 mM ciprofibrate for 1, 4, 8, 12 and 24 h. The mRNA sizes indicated are: FAOxase 3.8 kb, PBE 3.0 kb, and albumin 2.3 kb.

To investigate whether inhibition of protein synthesis abolishes the ciprofibrate-inducible increases in the levels of mRNAs of β-oxidation system genes, we treated hepatocytes for 8 h with 0.1 mM ciprofibrate in the presence of 10 μg/ml cycloheximide. At this concentration, although cycloheximide caused 93% inhibition of protein synthesis (data not presented), it did not interfere with ciprofibrate-induced FAOxase (figs 4A,5A), PBE (figs 4B,5B), THL (fig.5C) and catalase (fig.4C) mRNA accumulation.

The mechanism by which peroxisome proliferators increase the expression of genes responsible for the characteristic pleiotropic response is not known, but it may be mediated by specific binding protein(s) [2,6]. Evidence indicates that hepatocytes, irrespective of their location in the body, recognize and respond to peroxisome proliferators, thus exemplifying cell specific induction [2]. The results of in vivo and in vitro induction studies clearly show detectable increases in mRNA levels of β-oxidation genes in hepatocytes within 0.5–1 h after exposure to peroxisome proliferators [5,17]. The induction of transcription of β-oxidation genes in liver by ciprofibrate does not appear to require protein synthesis. Further, the inhibition of protein synthesis did not result in the superinduction of β-oxidation system mRNA levels. It is of interest to note that inhibition of protein synthesis does not superinduce the

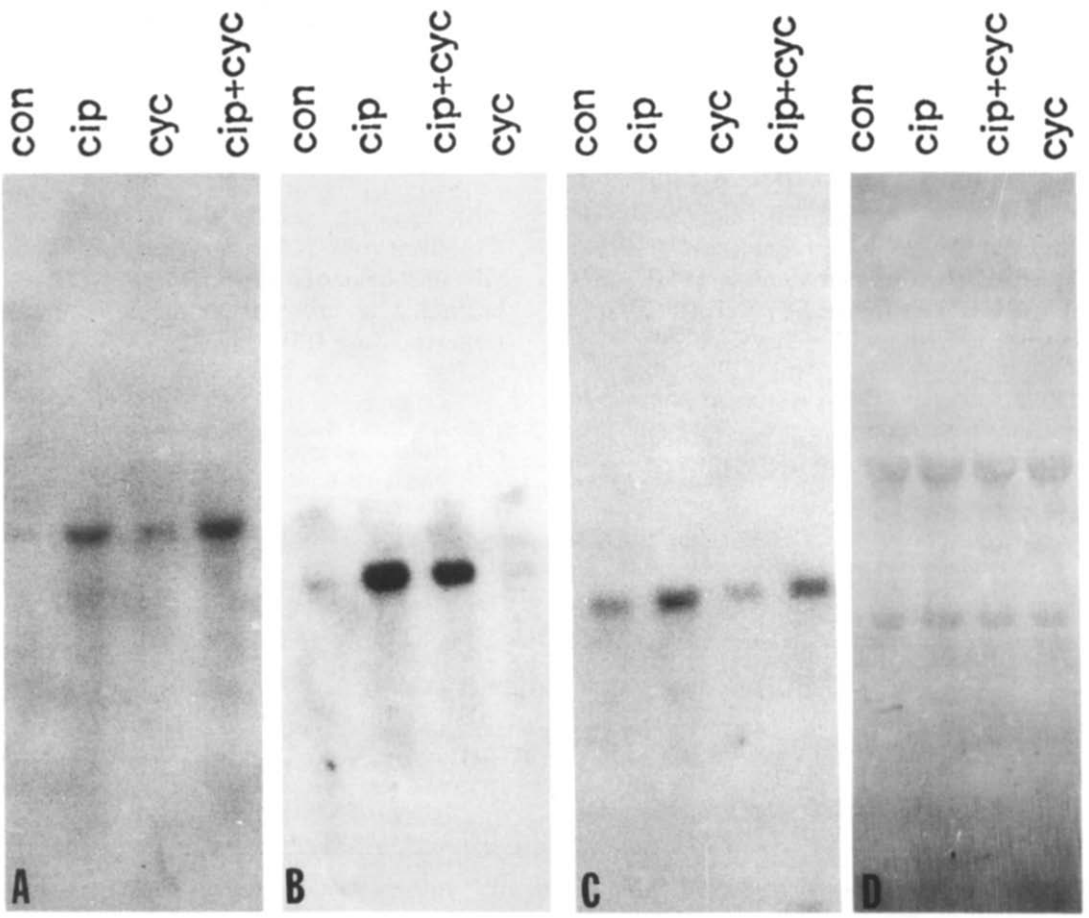

Fig.4. Effect of inhibition of protein synthesis by cycloheximide. Rat hepatocytes maintained in culture were exposed for 8 h to 0.1 mM ciprofibrate and/or 10 μg/ml cycloheximide. Total RNA (15 μg/lane) was used for this analysis. FAOxase (panel A); PBE (panel B); and catalase (panel C). Control (con); ciprofibrate (cip); ciprofibrate + cycloheximide (cip + cyc); cycloheximide (cyc). The mRNA sizes indicated are: FAOxase 3.8 kb, PBE 3.0 kb, and catalase 2.4 kb. The filter in panel B was stripped and stained with 2% methylene blue to indicate amount of RNA loaded (panel D).

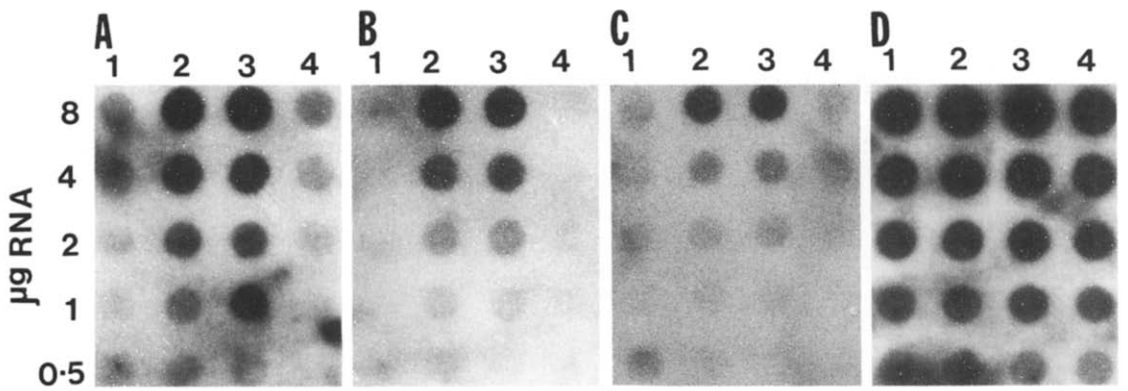

Fig.5. Dot-blot analysis of the effect of inhibition of protein synthesis on the induction of peroxisomal β-oxidation system mRNAs by ciprofibrate in rat hepatocytes in culture. Hepatocytes maintained in vitro were exposed to 0.1 mM ciprofibrate and/or 10 μg/ml cycloheximide for 8 h. Total RNA was purified, denatured with formaldehyde and applied to filters in the amounts indicated. Lanes: 1, no ciprofibrate, no cycloheximide; 2, ciprofibrate; 3, ciprofibrate and cycloheximide; 4, cycloheximide. The filters were hybridized with ^{32}P-labeled cDNAs for FAOxase (A), PBE (B), THL (C) and albumin (D).

transcription of certain estrogen responsive genes [19,20]. On the other hand, inhibition of protein synthesis enhances 2,3,7,8-tetrachlorodibenzo-*p*-dioxin mediated transcription of cytochrome *P*1-450 gene [21]. The results of the present study suggest that the action of the peroxisome proliferator and its postulated receptor is sufficient for maximal induction of the β-oxidation genes and that labile repressor protein(s) may not play a modulating role. Additional studies are needed to elucidate the mechanism by which peroxisome proliferators regulate this primary response.

Acknowledgements: This work was supported by NIH R37 GM23750. We thank Kathleen Stenson for typing the manuscript.

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