# Sodium butyrate inhibits the expression of the human lecithin:cholesterol acyltransferase gene in HepG2 cells by a post-transcriptional mechanism

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**Abstract We have previously demonstrated that LCAT is downregulated by TGF-β and that the regulation is post-transcriptional and involves an increased rate of RNA degradation. Sodium butyrate affects the expression of several liver-specific genes including some whose levels are altered during an acute-phase response. We have investigated the effect of sodium butyrate on LCAT activity and mRNA levels in HepG2 cells. Both the LCAT mRNA level and activity were reduced in a dose- and time-dependent manner. The reduction of LCAT mRNA levels was not, however, due to an increased degradation of processed mRNA. The transcriptional activity of the LCAT gene as seen in run-on experiments was not affected by sodium butyrate, whereas the total level of LCAT transcripts was reduced. Thus, LCAT activity and mRNA level in HepG2 cells are decreased by sodium butyrate treatment by a post-transcriptional mechanism, most likely involving increased degradation of pre-mRNA.**



*Key words:* Apolipoprotein A-I; Acute-phase proteins; HepG2 cells; Post-transcriptional regulation

## 1. Introduction

Sodium butyrate is a short-chain fatty acid produced by bacterial fermentation in the mammalian colon [1] where it inhibits cell proliferation and stimulates cell differentiation [2]. It affects many physiological processes in cultured mammalian cells, among these are the transcriptional activation of previously inactive genes and also the inactivation of a few active genes [3], reversible differentiation of cells [4] and blocking of cells in the $G_1$ phase of the cell cycle [5]. It has been reported that sodium butyrate can induce hypomethylation of genomic DNA in some cell systems [6] but also cause hypermethylation in others [7]. Additionally, sodium butyrate has been shown to suppress deacetylation of histones [8].

The differentiation-promoting effects of sodium butyrate have been demonstrated in several cell lines including rat and human hepatoma cells [9]. Sodium butyrate affects the expression of several liver-specific genes, among them proteins whose levels also are altered during an acute-phase response [10]. Treatment with sodium butyrate gives rise to changes in the chromatin structure and also to altered nuclear protein phosphorylation and methylation of proteins as well as DNA [11]. The exact mechanism by which sodium butyrate causes alteration in gene expression in each case, however, is not known.

The human lecithin:cholesterol acyltransferase (LCAT) is supposed to play a crucial role in the reverse transport of cholesterol from peripheral tissues [12] by catalyzing the esterification of cholesterol in high density lipoprotein (HDL) particles, thus creating a concentration gradient for the flux of cholesterol from cell membranes into HDL. Recently, we and others have shown that LCAT belongs to a group of proteins that affect lipid and lipoprotein metabolism during the acute-phase response [13,14]. Apolipoprotein A-I (apo A-I) and cholesterol ester transferring protein (CETP) also participate in the reverse cholesterol transport [15,16] and the level of apo A-I is reduced during the acute phase response [17]. The human hepatoma derived cell-line HepG2 has been found to express various liver-specific proteins, including LCAT, apo A-I and CETP, and is therefore in many respects a useful model of the human hepatocyte [18–20]. Sodium butyrate increased the mRNA levels of both apo A-I and CETP in HepG2 cells [21,22]. Apo A-I is a strong activator of the esterifying reaction catalyzed by LCAT and contains a binding domain for the LCAT protein [23]. The regulation of LCAT transcription is not well understood. We have previously shown that its expression is downregulated by TGF-β through decreased mRNA stability [13]. By perturbing LCAT expression in HepG2 cells using sodium butyrate, further insight into these regulatory processes has been obtained.

## 2. Materials and methods

### 2.1. Materials

Fetal calf serum (FCS) was from Biological Industries (Kibbutz Beth Haemek, Israel) and Dulbecco's modified Eagle's medium (DMEM), L-glutamine and trypsin-EDTA from Whittaker (Walkerville, MD, USA). Sodium butyrate, calf thymus DNA, human serum albumin (HSA, essentially fatty acid free), actinomycin D, lecithin and cholesterol were from Sigma (St. Louis, MO, USA). Apo A-I was isolated from human plasma [24]. Dynabeads were from Dynal (Oslo, Norway). [4-$^{14}$C]Cholesterol was from Amersham, UK. MagnaCharge membranes (MSI, Westboro, MA, USA) were used for hybridization.

### 2.2. Cell culture and treatment

The human hepatoma-derived cell line HepG2 (ATCC HB8065) was grown in DMEM with 10% fetal calf serum and maintained in humidified 5% $CO_2$, 95% air at 37°C. For experiments the cells were plated in 10 cm dishes at a density of $2\times10^6$ and allowed to grow for 4 days to reach about 75% confluence. The cells were then given fresh medium containing various concentrations of sodium butyrate (0.5–5 mM). The medium was removed after 24 h and the cells were washed with PBS followed by incubation for another 24 h in serum-free DMEM supplemented with the same concentrations of sodium butyrate. When indicated the cells were incubated with actinomycin D (5 μg/ml) in serum-free medium for 30 min prior to the addition of sodium butyrate.

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*Abbreviations:* LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; CETP, cholesterol ester transferring protein; apo A-I, apolipoprotein A-I


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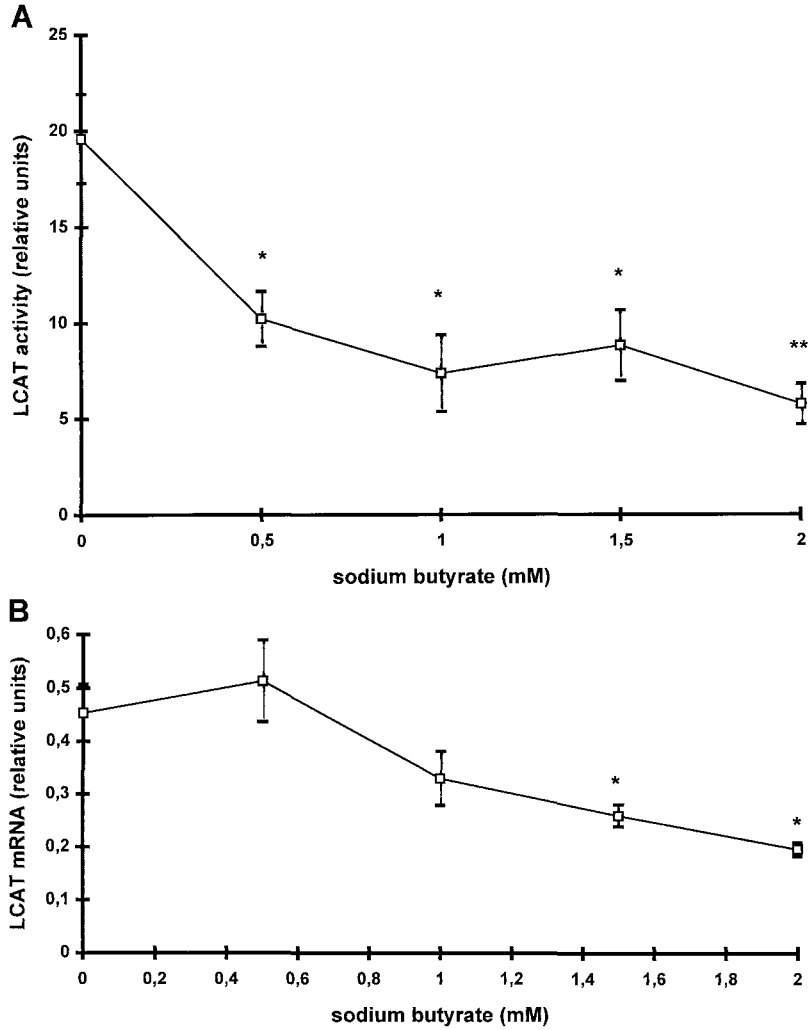


Fig. 1. Dose-response curve for the effect of sodium butyrate on LCAT activity (A) and mRNA level (B) of HepG2 cells. Cells were treated with various concentrations of sodium butyrate (0–2 mM) for 24 h in the presence of DMEM with 10% FCS and further incubated at the same concentration of sodium butyrate in medium without serum for another 24 h. LCAT activity and mRNA levels were measured as described in Section 2. Data are presented as mean ± S.E.M. ($n = 3$, each in duplicate), $^{*}P < 0.05$ and $^{**}P < 0.005$ vs. control.

*2.3. LCAT activity*

LCAT activity was measured in medium from HepG2 cells by the addition of radiolabelled proteoliposomes (apo A-I:lecithin:cholesterol, 0.8:250:12.5 molar ratio) to medium using a modification of the method by Chen and Albers [25]. The final reaction volume was 1 ml. The substrate was preincubated for 20 min at 37°C with HSA (final concentration 0.5%). Medium and 2-mercaptoethanol (final concentration 5 mM) were added, the mixture was incubated for 24 h at 37°C and the enzyme activity was assayed by measuring the conversion of cholesterol to cholesterol ester after separation by thin-layer chromatography. Preliminary experiments showed that the conditions used allowed zero order kinetics. Less than 15% of the substrate was converted.

*2.4. RNA analysis*

LCAT mRNA was isolated directly from PBS-washed cultured cells using oligo(dT) Dynabeads according to a procedure modified from that of Jakobsen et al. [26]. The cells were lysed and homogenized in buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT) by shearing three times through a 21 gauge syringe. The RNA was blotted onto MagnaCharge membranes, hybridized (0.7×SSC, 1×SPEP, 1×Denhardt) and washed (0.7×SSC, 1% SDS) at 68°C. LCAT and apo A-I mRNA levels were determined using human LCAT and apo A-I cDNA probes [27,28]. To adjust for the amount of mRNA on the filters a cDNA probe for human GAPDH [29] was used. Random priming was used to label the DNA probes with [α-$^{32}$P]dATP and [α-$^{32}$P]dCTP. Linearly exposed autoradiograms were scanned in a Shimadzu CS-9000 densitometer.

*2.5. Nuclear run-on transcription assay*

Nuclei were prepared as described by Graves et al. [30] with some modifications. Untreated and treated (24 h) HepG2 cells from 10 cm dishes were scraped into ice-cold PBS, centrifuged and resuspended in 50 μl PBS. Cells were lysed by the addition of 450 μl Nonidet P-40 buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40). After 5 min incubation on ice and centrifugation at 265×$g$ and 4°C, the sedimented nuclei were resuspended in storage buffer (50 mM Tris-HCl pH 8.0, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 70 mM KCl, 2.5 mM DTT).

Transcription assays were performed as described by Bartalena et al. [31] and Baumhueter et al. [32]. Nuclei ($1–3 \times 10^6$) were incubated in the storage buffer in the presence of 0.62 mM rATP, 0.31 mM rGTP, 0.31 mM rCTP, 80 U RNasin and 250 μCi [α-$^{32}$P]UTP (800 Ci/mmol) at 27°C in a total volume of 400 μl. After 20 min 3 μl 10 mM UTP was added and the reaction mix was further incubated at 37°C for 15 min. The reaction was stopped by adding DNase I (1 mg/ml) and 200 μg tRNA. The labelled RNA was purified using proteinase K, phenol/chloroform extractions and ethanol/ammonium acetate precipitation. The labelled RNA species were then hybridized for 3 days onto nylon membranes with immobilized cDNAs for apo A-I, LCAT, GAPDH and pBR322 DNA as control. Hybridizations were performed at 42°C for 72 h in 30% formamide, 300 mM

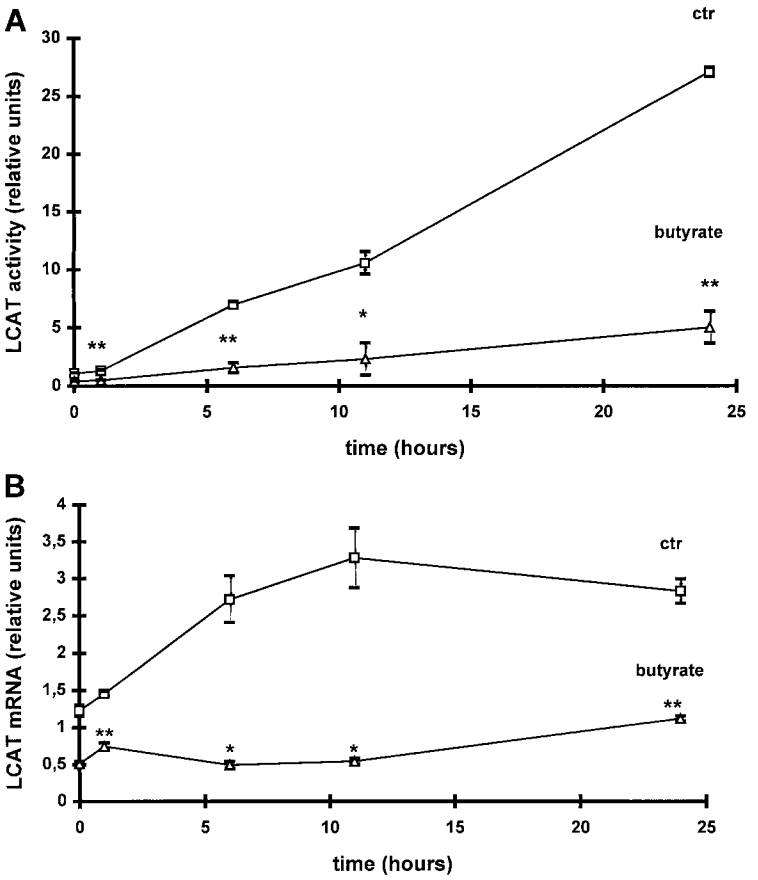


Fig. 2. Time course of the effect of sodium butyrate on LCAT activity (A) and mRNA level (B) of HepG2 cells. Cells were treated with 2 mM sodium butyrate in serum-free medium after a 24 h preincubation in medium with 10% FCS and the same concentration of sodium butyrate. At the indicated times, samples of cells and medium were analyzed for LCAT activity and mRNA levels as described in Section 2. Data are presented as mean ± S.E.M. ($n=4$, each in duplicate), $^{*}P<0.05$ and $^{**}P<0.005$ vs. control.

NaCl, 10 mM EDTA, 100 mM Tris-HCl pH 7.2, 0.2% SDS, 0.4% Ficoll and 100 μg/ml tRNA. Filters were washed 2×15 min at 42°C in 10 mM Tris-HCl pH 7.5, 300 mM NaCl, 2 mM EDTA and 0.1% SDS and 2×15 min at 65°C in 10 mM Tris-HCl pH 7.5, 60 mM NaCl, 0.1 mM EDTA, 0.1% SDS followed by autoradiography at −70°C. Cold nuclear run-on transcription assays were performed similarly using only unlabelled nucleotides. The purified nuclear RNA was then separated on a denaturing agarose gel, blotted onto MagnaCharge membranes and hybridized using labelled LCAT and GAPDH cDNA as probes.

## 3. Results

### *3.1. Dose dependence and time course of the effect of sodium butyrate on LCAT activity and LCAT mRNA*

To investigate the influence of sodium butyrate on expression of the LCAT gene, HepG2 cells were treated with various concentrations of sodium butyrate for different time intervals. Kaptein et al. [21] have shown that sodium butyrate induces an increase in the level of mRNA for apo A-I as well as in the secretion of this protein after a lag period of approximately 24 h. Based on their results we incubated HepG2 cells with 0.5–2 mM sodium butyrate for 24 h in medium containing serum followed by a further incubation in serum-free medium at the same butyrate concentrations for another 24 h. (Incubation in serum-free medium beyond 24 h in itself affected LCAT levels.) Control cells were incubated for 24 h in medium containing serum followed by incubation in serum-free medium for the same interval.

Sodium butyrate (2 mM) caused a 5-fold decrease in the LCAT activity after 48 h (Fig. 1A). This was accompanied by a decrease in the level of LCAT mRNA to about 35% of the control value (Fig. 1B). A significant decrease in LCAT activity was seen at concentrations of 0.5 mM sodium butyrate or higher ($P<0.05$); for LCAT mRNA a significant decrease was only observed at or about 1.5 mM ($P<0.05$).

The time dependence of the sodium butyrate effect was investigated by treating HepG2 cells with 2 mM sodium butyrate for 1, 6, 11 and 24 h in serum-free medium. After 6 h LCAT activity in the medium from sodium butyrate-treated cells had declined to 27% of control values. In contrast to the increase of apo A-I mRNA observed by Kaptein et al. [21], we detected a decline in LCAT mRNA to about half of the control value after 24 h (Fig. 2B). This level remained unchanged during the next 24 h. LCAT activity in the medium from the butyrate-treated cells had decreased to about 16% of that in medium from the control cells (Fig. 2A).

### *3.2. Effect of sodium butyrate on mRNA half-life*

The effect of sodium butyrate was then studied in experiments where RNA synthesis was inhibited by actinomycin D as described. HepG2 cells were grown in the absence and presence of sodium butyrate. Actinomycin D was added 30 min before sodium butyrate. From these cultures mRNA was isolated over a time course of 48 h. LCAT mRNA from un-

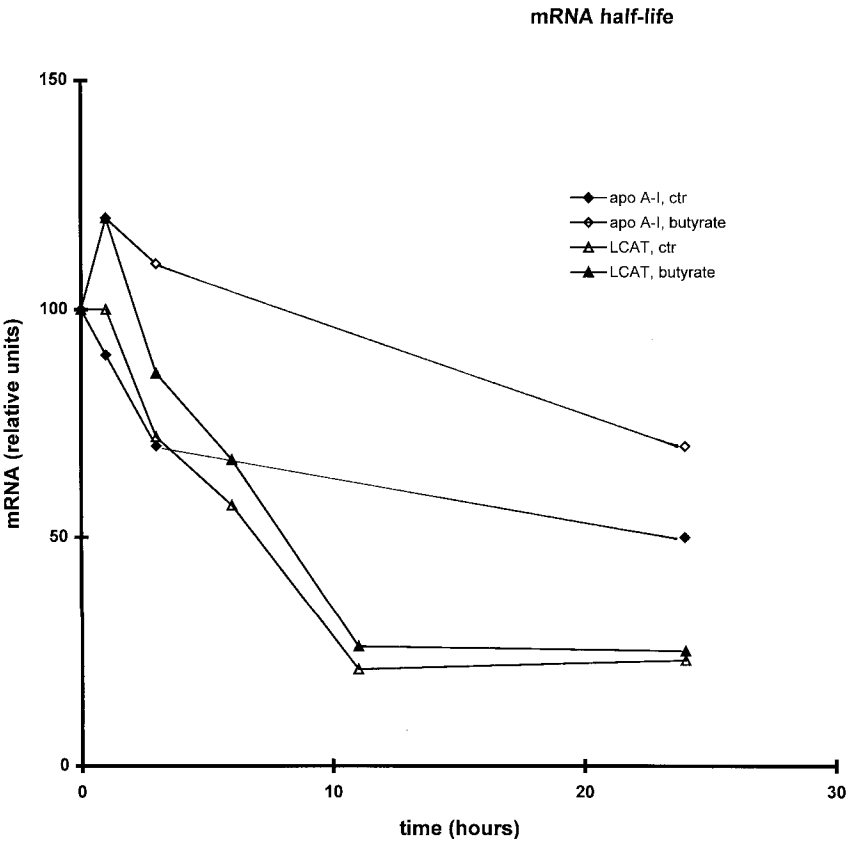


Fig. 3. Effect of sodium butyrate on LCAT and apo A-I mRNA half-life in HepG2 cells. The cells were treated with actinomycin D (5 μg/ml) in the absence or presence of sodium butyrate (2 mM). mRNA was isolated at the indicated times (0, 1, 3 and 24 h) and analyzed as described in Section 2. Data are given in percent of the value for mRNA at the start of the experiment.

treated and sodium butyrate-treated cells appeared to decay equally slowly (Fig. 3), with a half-life of approximately 8 h. Also apo A-I mRNA decayed at essentially the same rate in butyrate-treated and control cells (Fig. 3).

### 3.3. *Effect of sodium butyrate on the transcriptional activity of the LCAT gene*

To determine whether the decrease in LCAT mRNA levels in HepG2 cells after treatment with sodium butyrate was caused by decreased transcription of the LCAT gene, run-on experiments were performed on nuclei from untreated HepG2 cells and from cells treated for 24 h with sodium butyrate. In these assays, only transcripts which are already initiated are elongated, thus giving an accurate measure of the level of transcription at the time of the nuclear isolation. In such run-on experiments there was no change in the transcription rate of the LCAT gene after treatment with sodium butyrate (Fig. 4) even though sodium butyrate significantly reduced the level of LCAT mRNA seen by Northern blot analysis (Figs. 1B and 2B). We therefore decided to visualize the LCAT pre-mRNA levels by cold run-on assays, isolating nuclear RNA and running it out on a denaturing gel, blotting and hybrid-

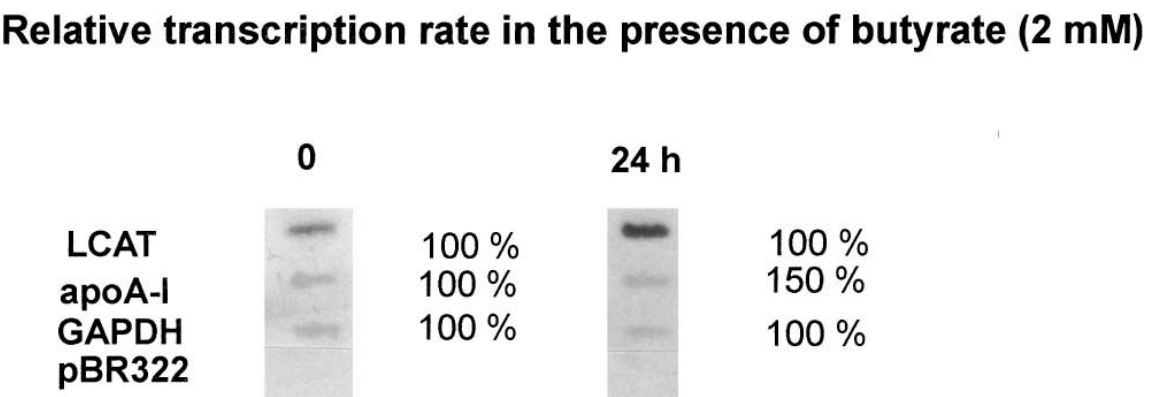


Fig. 4. Effect of sodium butyrate on the transcriptional activity of the LCAT and apo A-I genes. Relative transcription rates were determined in nuclei from HepG2 cells untreated or treated with 2 mM sodium butyrate for 24 h. Nuclei were isolated and run-on assays performed as described in Section 2. Data are given in percent of the transcription rate of control nuclei and represent the means of two independent run-on assays.

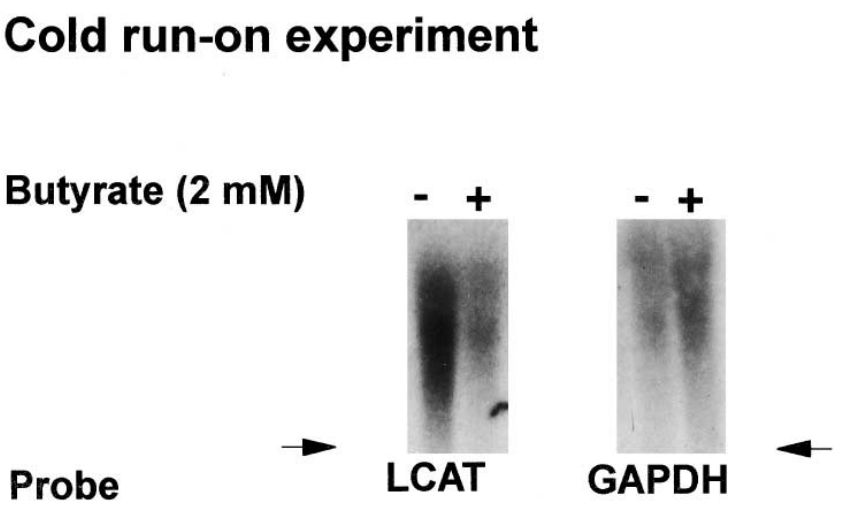


Fig. 5. Effect of sodium butyrate on total LCAT RNA transcripts in HepG2 cells as observed in cold run-on assays. Nuclei from cells untreated or treated with sodium butyrate (2 mM) were treated as described for run-on assays in Section 2. Total RNA was isolated and analyzed by Northern blots using LCAT and GAPDH cDNAs as probes. Arrows indicate the position of mature LCAT and GAPDH mRNA.

izing with labelled LCAT and GAPDH probes. As expected, a 'haze' was seen of RNA molecules of 1.5–4.2 kb, representing LCAT pre-mRNA at various stages of processing. Control hybridizations with GAPDH gave expected sizes (1.3–5.3 kb). A marked difference was seen between nuclear RNA samples from butyrate-treated and control cells, showing that LCAT transcripts, although initiated with equal frequency in treated and control cells (Fig. 4), was degraded more rapidly under butyrate treatment (Fig. 5).

Apo A-I mRNA is enhanced in HepG2 cells after treatment with sodium butyrate [21]. This increase is apparently partly due to an increased transcription of the apo A-I gene, since the transcriptional rate of the apo A-I gene after treatment with 2 mM sodium butyrate for 24 h increased approximately 50% (Fig. 4).

## 4. Discussion

The human hepatoma-derived cell line HepG2 retains many characteristics of the normal differentiated hepatocyte. It synthesizes and secretes apolipoproteins [19]. Also LCAT is secreted and LCAT mRNA is observed in Northern blot analysis [18,33]. The HepG2 line therefore constitutes a suitable model system to study expression of the LCAT gene. Treatment of HepG2 cells with sodium butyrate led to a decrease in LCAT expression. We have examined this effect of sodium butyrate on LCAT expression and its underlying causes. The decrease in LCAT mRNA levels was dose- and time-dependent with respect to butyrate treatment and accompanied by lowered LCAT activity in the culture medium. Sodium butyrate did not alter the rate of degradation of processed LCAT mRNA. LCAT expression was decreased by a post-transcriptional mechanism since the transcription rate as measured in run-on experiments was unaltered when butyrate-treated and control cells were compared. We conclude that the reduced steady-state LCAT mRNA level must be due to events taking place in the interval between transcription and degradation of processed mRNA. Evidence rendering further support for this hypothesis comes from the cold run-on experiments. When total nuclear RNA was subjected to agarose electrophoresis, blotted and hybridized to a LCAT probe, a smear of RNA molecules of apparent sizes approximately 1.5–4.2 kb is observed, as expected for LCAT pre-mRNA consisting of molecules at all stages of processing. In nuclear RNA from butyrate-treated cells there is a significant and clearly visible reduction in the amount of such RNA available for hybridization to the LCAT probe (Fig. 5). These data indicate that the action of butyrate in regulating LCAT mRNA levels has to do with regulation of pre-mRNA processing and stability and that butyrate induces a more rapid breakdown of pre-mRNA which is not fully processed.

We have previously reported that LCAT mRNA and activity were downregulated by a post-transcriptional mechanism when HepG2 cells were treated with TGF-β [13]. The present data suggest that TGF-β and sodium butyrate act via similar mechanisms in downregulating LCAT activity. It has been reported that sodium butyrate can induce alterations in DNA methylation [6,7]. Control experiments (Skretting, unpublished) showed no differences in the pattern of cleavage of LCAT genomic DNA (including the promoter region) by a number of methylation-sensitive restriction enzymes.

Apo A-I is the most efficient activator of the LCAT reaction in vitro. In vivo, apo A-I is a part of the discoidal, nascent HDL particle which takes up unesterified cholesterol from cell membranes. This cholesterol is esterified by LCAT and the HDL particle is transformed into a spherical, mature particle with a cholesterol ester core. Apo A-I interacts with the LCAT protein to activate the enzyme through a specific domain in the apo A-I protein. Patients with various forms of apo A-I deficiency have lowered LCAT activity in plasma and patients with familial LCAT deficiency or fish-eye disease have lowered amounts of apo A-I in plasma [34–36]. These findings may suggest that there are common elements in the regulation of the expression of the LCAT and apo A-I genes. For apo A-I mRNA the regulation by TGF-β seemed to be both transcriptional and post-transcriptional. Sodium butyrate has been reported to stimulate the synthesis of apo A-I in HepG2 cells [21]. Our results show that this is partly caused by an increased rate of apo A-I gene transcription (and possibly also by an increased rate of pre-mRNA processing) in the presence of an essentially unaltered apo A-I mRNA stability.

In summary, the present study demonstrates that sodium butyrate downregulates the expression of the LCAT gene in HepG2 cells by a post-transcriptional mechanism, most likely by increased degradation of pre-mRNA, whereas apo A-I expression is upregulated, at least in part through an increased transcription rate.

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