FEBS Letters 380 (1996) 204–207 FEBS 16645

Insulin- and polyunsaturated fatty acid-responsive region(s) of rat ATP citrate-lyase gene promoter

Hitomi Fukuda[a], Nobuko Iritani[a,*], Akihiko Katsurada[a], Tamio Noguchi[b]

[a]*Tezukayama Gakuin College, 4-cho, Harumidai, Sakai, Osaka 590-01, Japan.*
[b]*Department of Biochemistry, Fukui Medical School, Fukui 910-11, Japan*

Received 19 December 1995

Abstract To investigate the regulatory DNA sequences required for insulin-stimulation of the ATP citrate-lyase (ACL) gene as well as for polyunsaturated fatty acid (PUFA)-suppression of this gene, primary cultured hepatocytes were transfected with plasmids containing the 5′-flanking sequence of the rat ACL gene fused to the chloramphenicol acetyltransferase (CAT) gene. Sequences from −861, −194 or −104 to +128 of the ACL gene directed an increase in CAT activity in hepatocytes when insulin was added to the medium containing either glucose or pyruvate. The CAT activities stimulated by insulin were reduced by the addition of PUFA, in accordance with the responses on the endogenous ACL gene expression. Further deletion to −20, however, resulted in loss of the responses. The results suggest that the region from −104 to −20 of the ACL gene is responsible for regulation due to insulin and PUFAs. In particular, the region from −61 to −49 of the ACL has sequence similarity to the insulin-responsive regions of fatty acid synthase and acetyl-CoA carboxylase.

Key words: ATP citrate-lyase gene; Response region; Insulin; Polyunsaturated fatty acids

1. Introduction

ATP-citrate lyase (ACL) (EC 4.1.3.8) catalyzes the formation of acetyl-CoA and oxaloacetate from citrate and CoA with a concomitant hydrolysis of ATP to ADP and phosphate. The product, acetyl-CoA serves several important biosynthesis pathways, including lipogenesis and cholesterogenesis. Thus, ACL is considered as one of lipogenic enzymes. We previously reported that the gene expression of ACL in rat liver was increased by a fat-free/high-carbohydrate diet and decreased by fat-feeding or in fasted and diabetic states, in a similar manner to other lipogenic enzymes [1,2]. The gene expression was primarily regulated at transcriptional step [1].

Although transfection analyses using primary cultured hepatocytes demonstrated the presence of insulin-regulated *cis*-acting elements of fatty acid synthase gene [3,4], the insulin response element has not been found in ACL gene. Moreover, the presence of PUFA-regulated *cis*-acting elements has been reported in the proximal promotor regions of genes of L-type pyruvate kinase and S14, which gene expression is coordinately regulated with lipogenic enzymes by nutritional and hormonal manipulation [5,6]. However, little is known about the DNA sequences involved in expression of ACL. In order to investigate ACL gene expression, transfection system using cultured primary hepatocytes of rats was utilized in the present study.

2. Materials and methods

2.1. Materials

Restriction endonucleases and other enzymes were purchased from Takara Shuzo. The sequence kit, luciferase assay kit and acetyl-CoA were from Applied Biosystems, Toyo Ink and Sigma, respectively. Williams' medium E was purchased from Flow Laboratories. Other culture media were obtained from Nissui Seiyaku. [^{14}C]Chloramphenicol (2.22 GBq/mmol) and [α-^{32}P]dCTP (111 TBq/mmol) were from ICN. Lipofectin reagent was from Life Technologies.

2.2. Plasmid constructs

Plasmid pUC0cat, a promoter-less CAT vector, and pUC2cat, a CAT vector with simian virus 40 early enhancer and promotor were used as negative and positive controls, respectively [7]. Plasmid pactL, a luciferase vector containing β-actin enhancer and promoter, was used as an internal control to normalize for variations in transfection efficiency [8].

An ACL genomic clone was obtained by screening rat EMBL3 genomic library (Clontech) with rat ACL cDNA [1]. We sequenced the region from −2394 to +128 of this gene and found that sequences of this region coincided with those reported by Kim et al. [9]. ACLcat2394 was constructed by ligating a *Xho*I/*Hin*dIII fragment (−2394 to +128) of the rat ACL gene into *Sal*I/*Hin*dIII site of puC0cat.

Deletion of the ACLcat2394 plasmid was carried out using an exonuclease III. The fragments obtained were ACLcat2268, ACLcat1945, ACLcat1780, ACLcat1517, ACLcat1375, ACLcat1081, ACLcat861, ACLcat537, ACLcat194, ACLcat104 and ACLcat20. These CAT constructs have 3′ endpoint at +128 and 5′ endpoint at −2268, −1945, −1780, −1517, −1375, −1081, −861, −537, −194, −104 and −20, respectively, relative to the transcription start site of the rat ACL gene. The sequence of these inserts was verified by dideoxy sequencing, using a sequenase kit [10].

2.3. Primary hepatocyte culture and transfection

Male Wistar rats (200–250 g) maintained on a stock diet (Oriental Koubo, MF) were fasted for 16 h before experiments. The hepatocytes were isolated by the collagenase perfusion method [11,12] and plated at a density of 3×10^6 cells/60 mm Primaria culture dish (Falcon). After a 6 h attachment period, medium was replaced with modified Williams' E media (lacking methyl linolate) supplemented with 5 mM glucose, 26 mM sodium bicarbonate, 2 mM glutamine and 1 μM dexamethasone, and then mixtures of 2 μg of pactL and 18 μg each of CAT constructs were transfected into hepatocytes using lipofectin for 16 h. Subsequently, cells were cultured for 48 h in experimental media (with 100 μg/ml streptomycin and 100 U/ml penicillin) containing either 20 mM glucose or pyruvate with or without 0.1 μM insulin. When included, 0.1 mM fatty acids was added. All transfections were performed at least twice.

2.4. CAT and luciferase assay

The cells were incubated for 48 h after transfection, harvested and lysed by sonication. Then 20 μl of the supernatant from each sample

*Corresponding author. Fax: (81) (722) 92-2135.

Abbreviations: ACL, ATP citrate-lyase; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; CAT, chloramphenicol acetyltransferase; PUFA, polyunsaturated fatty acids; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

SSDI S0014-5793(96)00031-2

were assayed for luciferase [13] using a kit. Normalized amounts of the cell extracts by luciferase activity were used for CAT assays after heating at 60°C for 10 min [14,15]. Acetylated and nonacetylated forms of [^{14}C]chloramphenicol were determined by scintillation counter and the percentages of the acetylated forms were calculated. All transfections were performed at least three times in duplicate.

2.5. Preparation of RNA and dot blot hybridization assay

Total cellular RNA was isolated from cultured cells by the method of acid guanidine thiocyanate-phenol-chloroform extraction [16]. The cDNA species of ACL were cloned as described previously [1]. The genomic clone of rat rRNA was obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan). About 1 kb *Bam*HI/*Eco*RI fragment of this clone was isolated and used as a probe for 18S rRNA. The cDNAs were labeled by a multiprimer DNA labeling system kit (Amersham, Buckinghamshire, UK) using [α-^{32}P]dCTP. To measure the mRNA concentration of the enzyme, the total RNA (20 μg) was denatured with formaldehyde at 65°C for 15 min, spotted on nylon filter, and then radiated with UV light for 5 min. The filter was prehybridized and then hybridized as described previously [17]. Relative densities of the hybridization signals were determined by scanning the autoradiograms at 525 nm and normalized to the values of the 18S rRNA.

3. Results and discussion

3.1. Effects of insulin and PUFA on ACL mRNA concentration in cultured hepatocytes

We previously found that the mRNA inductions of lipogenic enzymes became maximum after 16–24 h in cultured hepatocytes of rat and the maximum levels still continued after 48 h [18]. Therefore, to examine the effects of insulin, glucose and PUFA on the endogenous ACL gene expression, the mRNA concentrations were measured after 48 h incubation of hepatocytes. As shown in Table 1, the addition of insulin clearly enhanced the mRNA concentrations of ACL in the presence of pyruvate or glucose, indicating that glucose is not necessarily required for the effect of insulin on this gene. The insulin stimulation was markedly suppressed by arachidonic acid (20:4).

3.2. Insulin and PUFA regulation of transfected ACL gene

To define the ability of ACL sequences to direct insulin-stimulated expression, the constructs between −2394 and +128 of the ACL gene were fused to the CAT gene and introduced into primary hepatocytes. The cells were cultured in the presence of 20 mM pyruvate or glucose with or without insulin for 48 h, and then extracts were assayed for CAT activity.

Table 1
Effects of insulin and PUFA on ACL mRNA concentrations in cultured hepatocytes

Additions	mRNA concentration (-fold)
Pyruvate	0.50 ± 0.10
Pyruvate+insulin	1.47 ± 0.27
Pyruvate+insulin+20:4	0.55 ± 0.12
Glucose	0.50 ± 0.13
Glucose+insulin	1.00
Glucose+insulin+20:4	0.43 ± 0.12

After 16 h preculture without serum and hormones in 5 mM glucose, hepatocytes were incubated with or without 0.1 μM insulin for 48 h in the presence of 20 mM pyruvate or glucose. When included, 0.1 mM arachidonic acid (20:4, n-6) was added. The mRNA concentrations were normalized to the value glucose + insulin. Mean + S.D. (n = 5–6).

Table 2
Expression of ACLcat fusion genes in rat hepatocytes

	Relative CAT activity (%)
ACLcat2394	99.6 ± 21.8
ACLcat2268	83.7 ± 15.9
ACLcat1945	108 ± 33.5
ACLcat1780	93.7 ± 34.3
ACLcat1517	81.2 ± 29.3
ACLcat1375	99.5 ± 31.0
ACLcat1081	88.6 ± 4.18
ACLcat861	89.5 ± 18.4
ACLcat537	105 ± 26.8
ACLcat194	88.7 ± 31.0
ACLcat104	132 ± 29.3
ACLcat20	9.20 ± 4.18
pUC0cat	1.01 ± 0.04

The constructs of the ACL gene fused to the CAT gene were introduced into primary hepatocytes. These ACLcats have 3′ endpoint at +128 and 5′ endpoint at −2394 to −20. The hepatocytes were incubated with 0.1 M insulin and 20 mM glucose for 48 h after transfection, harvested and assayed for CAT activities. The percentages of acetylated forms of [^{14}C]chloramphenicol were determined as the CAT activities for each sample. The relative CAT activities of the deletion constructs are expressed as percentages of that of pUC2cat. Means ± S.D. of 5–7 independent experiments are shown.

As shown in Table 2, the ACLcats having 3′ endpoint at +128 and 5′ endpoint at −2394 to −104 exhibited similar CAT activities in the presence of glucose and insulin. However, the CAT activities of ACLcat20 markedly decreased. Fig. 1 shows one of the typical results of autoradiograms for the CAT activities of ACLcat861, ACLcat194, ACLcat104 and ACLcat20. Insulin induced the activities of ACLcats861,194,104 in the presence of glucose or pyruvate. The insulin-stimulation in cells cultured with pyruvate were found to have a similar (or rather higher in ACLcat104) CAT activity to the stimulation in cells grown with glucose. Thus, glucose was not necessarily required to the insulin activation of the CAT activities. In contrast to ACL, the other insulin-responsive genes, such as L-type pyruvate kinase and S14 required the presence of both insulin and glucose in cultured hepatocytes [19,20]. Thus, sequence elements located within −104 bp of the transcriptional start site are sufficient to confer insulin-stimulated expression. Further deletion to −20, however, resulted in loss of this response, indicating that sequences responsible for insulin is located between −104 and −20 of ACL gene.

The insulin stimulation of the CAT activities of ACLcat861, ACLcat194 and ACLcat104 was reduced in arachidonic acid (20:4, n-6) treated cells, representing a 60% decline in ACL CAT activity. This suggests that arachidonic acid inhibited endogenous ACL gene expression at the transcriptional level (Fig. 1). The arachidonic acid-suppression of the insulin stimulation in the CAT activities roughly paralleled the suppression of ACL mRNA concentrations. However, as the insulin stimulation of the CAT activity of ACLcat104 was very high (7-fold) in the presence of pyruvate, the stimulation was not completely suppressed by arachidonic acid. The upstream of −104 of ACL gene may have some sequences to disturb the insulin stimulation.

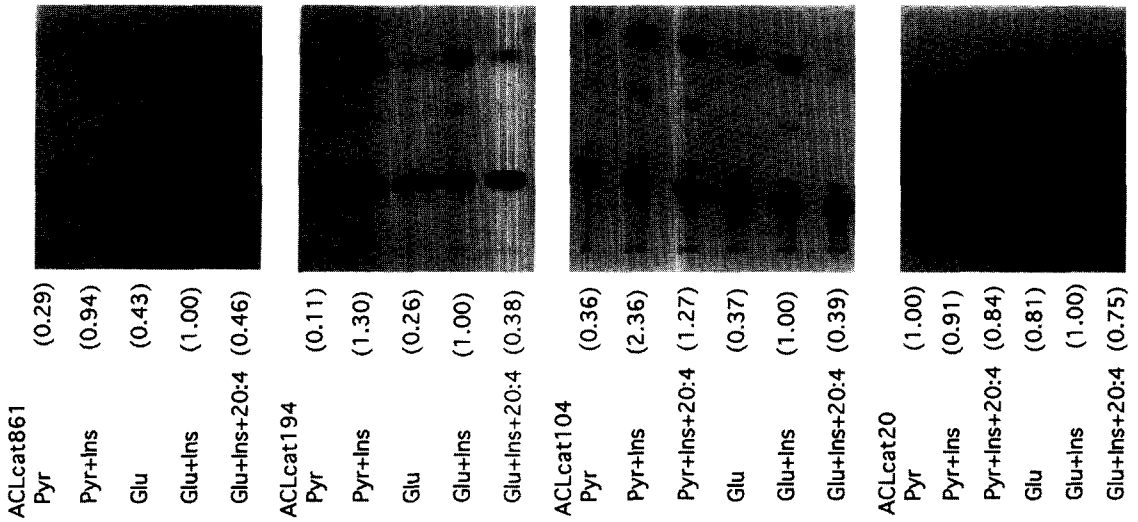

Fig. 1. The CAT activities of the constructs of ACL gene. The constructs of the ACL gene fused to the CAT gene (ACLcat861, ACLcat194, ACLcat104, ACLcat20) were introduced into primary hepatocytes. The hepatocytes were incubated with or without 0.1 μM insulin (Ins) for 48 h in the presence of 20 mM pyruvate (Pyr) or glucose (Glu). When included, 0.1 mM arachidonic acid (20:4, n-6) was added. One of the typical results is shown. The percentages of acetylated forms are normalized to the value for glucose+insulin in each ACLcat and shown as the relative CAT activities in parentheses.

The insulin stimulation of the CAT activities of ACLcat104 was not reduced in stearic acid (18:0) or oleic acid (18:1) treated hepatocytes, but reduced in arachidonic acid treated cells. Similar effects were seen when eicosapentaenoic acid (20:5, n-3) or linoleic acid (18:2, n-6) was substituted for arachidonic acid (Fig. 2). Both n-3 and n-6 PUFA appeared to suppress the insulin activation of ACL gene. Thus the region between −104 and −20 of the ACL gene is responsible for PUFA-repression as well as for insulin-stimulation of this gene. However, it is unknown if the responsive region of PUFA is different from that of insulin or not. Moreover, this does not necessarily mean that this region contains *cis*-acting element responsive to PUFA, since PUFA may somehow inhibit the insulin signaling pathway from the insulin receptor to the ACL gene [21,22].

Table 3 shows comparison of nucleotide sequences of insulin- and PUFA-responsive region(s) of the ACL gene with those of other insulin-responsive genes such as FAS, ACC and GAPDH [3]. These regions have some sequence similarity between nucleotides −74 to −66 of the ACL gene and nucleotides −454 to −446 (7/9) and −462 to −454 (7/9) of the insulin-responsive region of GAPDH gene [3]. The nucleotides −87 to −79 of the ACL gene have some similarity to nucleotides −54 to −46 of the FAS gene (6/9). The nucleotides −61 to −49 of the ACL gene have some sequence similarity, especially between nucleotides −160 to −148 of the insulin responsive region of ACC gene [23,24] (8/12 in −61 to −50 and 8/11 in −60 to −50). The similarity between nucleotides −60 to −49 of the ACL gene and nucleotides −54 to −43 of the FAS gene was 9 out of 12 and 8 out of 9 (−51 to −43). Thus, the result suggests that the region of −61 to −49 of the ACL gene is insulin-responsive. However, significance of these similarity remains to be determined. The PUFA responsive element has been reported in the promoter region of the L-type pyruvate kinase L gene [5]. It has some similarity to the FAS gene (Fukuda, Iritani, Katsurada and Noguchi, unpublished data) but very little similarity to the ACL gene.

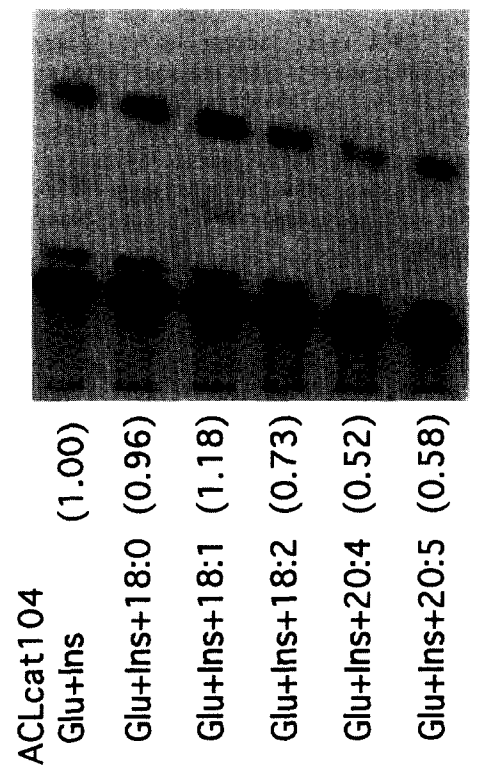

Fig. 2. Effect of fatty acid species on the CAT activities of the constructs of ACL gene. The construct of the ACL gene fused to the CAT gene (ACLcat104) was introduced into primary hepatocytes. The hepatocytes were incubated with 0.1 μM insulin (Ins) and 20 mM glucose (Glu) for 48 h, in addition of either 0.1 mM stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2, n-6), arachidonic acid (20:4, n-6) or eicosapentaenoic acid (20:5, n-3). One of the typical results is shown. The percentages of acetylated forms are normalized to the value for glucose+insulin and shown as the relative CAT activities in parentheses.

Table 3
Sequence comparison of postulated insulin- and PUFA-response element(s) of ACL, FAS, ACC and GAPDH genes

	Sequence		
ACL (-87/-49)	5'-GCCAGGCTGCATGGCCTGTGAGCTGATGGGGGGCGGGGA-3'		
	*** ** *	**** * **	* * *******
	GCCGCGCGG	GCCTCTCAG	GCCGCGCGGGGA
	FAS	GAPDH	FAS
	(-54/-46)	(-462/-454)	(-54/-43)
		**** ***	***** * **
		GCCTTTGAA	TGGGGTCGGAGGT
		GAPDH	
		(-454/-446)	**** *** *
			TGGGGTCGGAGGT
			ACC
			(-160/-148)

*Similar to nucleotide of the ACL gene.

References

[1] Fukuda, H., Katsurada, A. and Iritani, N. (1992) Eur. J. Biochem. 209, 217–222.
[2] Iritani, N. (1992) A review, Eur. J. Biochem. 205, 433–442.
[3] Moustaid, N., Sakamoto, K., Clarke, S., Beyer, R.S. and Sul, H.S. (1993) Biochem. J. 292, 767–772.
[4] Wolf, S.S., Hofer, G., Beck, K.-F., Roder, K. and Schweizer, M. (1994) Biochem. Biophys. Res. Commun. 203, 943–950.
[5] Liimatta, M., Towle, H.C., Clarke, S. and Jump, D.B. (1994) Mol. Endocrinol. 8, 1147–1153.
[6] Jump, D.B., Clarke, S., MacDougald, O. and Thelen, A. (1993) Proc. Natl. Acad. Sci. USA 90, 8454–8458.
[7] Takenaka, M., Noguchi, T., Inoue, H., Yamada, K., Matsuda, T. and Tanaka, T. (1989) J. Biol. Chem. 264, 2363–2367.
[8] Wang, Z., Takenaka, M., Imai, E., Yamada, K., Tanaka, T. and Noguchi, T. (1994) Eur. J. Biochem. 220, 301–307.
[9] Kim, K.-S., Park, S.-W., Moon, Y.-A. and Kim, Y.-S. (1994) Biochem. J. 302, 759–764.
[10] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[11] Seglen, P.O. (1976) Methods Cell Biol. 13, 27–83.
[12] Felgner, P.L., Gadek, T.R., Hold, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielson, M. (1987) Proc. Natl. Acad. Sci. USA 84, 7413–7417.
[13] Braiser, A.R., Tate, J.E. and Habener, J.F. (1989) Biotechniques 7, 1116–1121.
[14] Crabb, D.W. and Dixon, J.E. (1987) Anal. Biochem. 163, 88-92.
[15] Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell. Biol. 2, 1044–1051.
[16] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 163, 156–159.
[17] Katsurada, A., Iritani, N., Fukuda, H., Matsumura, Y., Nishimoto, N., Noguchi, T. and Tanaka, T. (1990) Eur. J. Biochem. 190, 427–433.
[18] Fukuda, H., Katsurada, A. and Iritani, N. (1992) J. Biochem. 111, 25–30.
[19] Shih, H.-M., Liu, Z. and Towle, H.C. (1995) J. Biol. Chem. 270, 21991–21997.
[20] Shih, H.-M. and Towle, H.C. (1994) J. Biol. Chem. 269, 9380–9387.
[21] Iritani, N., Hosomi, H., Fukuda, H. and Ikeda, H. (1995) Biochim. Biophys. Acta 1255, 1–8.
[22] Iritani, N. and Fukuda, H. (1995) J. Nutr. Sci. Vitaminol. 41, 207–216.
[23] Kim, K.-H. and Tae, H.-J. (1994) J. Nutr. 124, 1273S–1283S.
[24] Luo, X. and Kim, K.-H. (1990) Nucleic Acids Res.