

## Effects of glucose and insulin on insulin receptor gene expression

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### **Abstract**

We have investigated the effects of glucose, insulin and polyunsaturated fatty acids on the transcription of the insulin receptor (IR) gene, using IR(-618/-593) linked to a reporter gene in primary cultured hepatocytes of rats. When three copies of the nucleotides -618 to -593 of the IR promoter were transfected, the reporter activity was significantly increased in the presence of glucose and more increased in the presence of glucose/insulin. The glucose/insulin stimulation was inhibited by the addition of polyunsaturated fatty acids. These results were similar to those found earlier for the transcriptions of the fatty acid synthase, FAS(-57/-35), ATP citrate-lyase, ACL(-64/-41) and leptin(-101/-83) genes, which promoter sequences have a high similarity to IR (-618/-593). However, the IR gene was less responsive to insulin stimulation and more responsive to glucose stimulation than those. Mutational analysis of IR showed that the consensus Sp1 binding site from -618 to -593 is responsive to the glucose/insulin stimulation. Mutation of either one of the overlapping Sp1 binding sites (-618/-593) did not significantly affect the transcription. Gel mobility shift assays revealed that nuclear factor(s) from rat liver specifically formed complexes with the sequence of IR(-618/-593). By antibody supershift assays, the transcription factors Sp1 and Sp3 were found to bind with the IR gene. The DNA-protein complexes were common to the glucose/insulin-responsive regions of the IR, leptin, ACL and FAS genes, suggesting that these genes are relatively regulated.

### **Introduction**

Insulin is a key hormone regulating the control of glucose and lipid metabolism. The metabolic effects of insulin are initiated by the binding of insulin to the extracellular domain of insulin receptor (IR) within the plasma membrane of muscle and adipose and liver [1, 2]. The IR is a membrane protein that binds insulin and transmits a signal to the interior of the cell by activation of its tyrosine kinase activity [3]. The IR number can be altered by many physiological and

pathological factors, resulting in changes in cellular sensitivity to the actions of the hormone [1-5]. The cloning and sequencing of the human IR gene [6, 7] and its 5' flanking region [8-12] have facilitated the study of regulation of the IR synthesis. The promoter region of the human IR gene lacks a TATA and CAAT boxes, is extremely GC rich and contains seven GC boxes that are putative binding sites for mammalian transcription factor, Sp1 [8-12]. These reflect common features of promoters constitutively expressed housekeeping genes. Araki et al. [13] reported that the efficient expression of the human IR gene requires the binding of transcription factor Sp1 to four GC boxes located -618 to -593 base pairs upstream from the ATG translation initiation codon.

We previously mapped the sequences responsible for glucose/insulin-stimulation in the proximal promoter regions from -57 to -35 of fatty acid synthase (FAS) [EC 2.3.1.85] and from -64 to -41 of ATP citrate-lyase (ACL) [EC 4.1.3.8.] of rat liver [14, 15]. When two copies of a synthetic nucleotide probe of region -57 to -35 of the FAS gene were linked to a reporter gene and the resultant construct was used for transfection, the reporter gene activity was significantly increased in hepatocytes in response to glucose/insulin treatment and the glucose/insulin stimulation was suppressed by polyunsaturated fatty acid (PUFA), Sp1 and leptin [16]. The same results were observed with the -64 to -41 region of the ACL gene [17] and -101 to -83 of the leptin gene [18]. Nucleotides -57 to -35 of the FAS gene, -64 to -41 of the ACL gene and -101 to -83 of the leptin gene contain common binding sites for Sp1 proteins [16-18]. It is thus possible that nucleotides -618 to -593 of IR may be a glucose/insulin response element and Sp1 may be involved in the transcriptional regulation. The IR is expressed at low levels in all cell types but is expressed at much higher levels in the insulin target tissues: liver, adipose tissue and muscle [19].

In the present study, in order to make clear these possibilities, we have investigated the effects of glucose, insulin and PUFA on the transcription of the IR gene, using IR(-618/-593) linked to a reporter gene in primary cultured hepatocytes of rats.

## Materials and methods

**Materials** Restriction endonucleases and other enzymes were purchased from Takara Shuzo (Kyoto). The PGL3 promoter vector and dual luciferase assay system were purchased from Promega (Heidelberg). William's medium E was purchased from Flow Laboratories (Costa Mesa, CA) and other culture media from Nissui Seiyaku (Tokyo). [ $\gamma$ - $^{32}$ P]ATP (110GBq/mmol) and [ $\alpha$ - $^{32}$ P]dCTP (111Tbq/mmol) were from ICN (Costa, CA). Nylon filters (Hybond N) were purchased from Amersham. Antibodies against Sp1 and Sp3 were obtained from Santa Cruz

Biotech (Santa Cruz, CA). Recombinant mouse leptin was obtained from R & D systems (Minneapolis, MN). Lipofectin reagent was from Life Technologies (Palo Alto, CA)

**Plasmid constructs** Plasmid pRSVSp1 [20] was generously donated by Prof. Y. Fujii-Kuriyama (Touhoku University, Japan). Plasmid pRCMVSp3 [21] was generously donated by Prof. G. Suske (Philipps Universitat Marburg, Germany). The following single-stranded oligonucleotides were synthesized by GIBCO Ltd (Grand Island, N.Y.).

IR(-618/-593) : 5'-GATCGGGCGGGGCGGGGCGGGACCGGGCGG-3'

IR(-618/-593) mutants: See Table 1.

IR(-603/-593) : 5'-GATCGGACCGGGCGG-3'

Leptin(-101/-83) : 5'-AGCTGGGGCGGGAGTTGGCGCTC-3'

FAS(-57/-35) : 5'-GATCGTGGCCGCGCGGGGATGGCCGCG-3'

ACL(-64/-41) : 5'-GATCTGATGGGGGGCGGGGAGGAGCCCCG-3'

Sp1: 5'-ATTTCGATCGGGGCGGGGCGAGC-3'

Double-stranded oligonucleotides of IR(-618/-593) or IR(-618/-593) mutants were inserted into the PGL3 promoter. Three copies of these sequences were linked to the reporter gene.

**Primary cell culture and transfection** Male Wistar rats (200-250g) maintained on a standard diet (Oriental Koubo, MF, Tokyo) were fasted for 16 h before experiments. The hepatocytes were isolated by the collagenase digestion and the DNA reporter constructs were transfected into the cells, as described previously [22]. Subsequently, the cells were cultured for 48 h in experimental medium (with 100  $\mu$ g/ml streptomycin and 100 units/ml penicillin) containing 20 mM glucose with or without 0.1  $\mu$ M insulin. To some cultures, 0.1 mM arachidonic acid or 0.3  $\mu$ M recombinant mouse leptin was added. All transfections were performed at least three times in duplicate.

**Reporter gene assay** The cells were incubated for 48 h after transfection, and harvested. The luciferase reporter activity was assayed by the dual luciferase assay system from Promega according to manufacturer's instructions and measured with a Turner Design TD-20/20 luminometer [23]. The luciferase activity driven by the -618 to -593 region of the IR promoter was expressed as a percentage of the SV40 promoter-driven luciferase activity, which served as a positive control in every transfection experiment.

**Preparation of RNA and dot blot hybridization** Total cellular RNA was isolated from cell cultures by the acid guanidinium thiocyanate-phenol-chloroform extraction method [24]. Dot blot hybridization of total cellular RNA was performed as described previously [22]. IR cDNA was generously donated from Prof. Y. Ebina (Tokushima University) [7]. The genomic clone of rat

rRNA was obtained from Japanese Cancer Research Resources Bank. A *Bam*HI/*Eco*RI fragment of approximately 1 Kb was isolated from this clone and used as a probe for 18S rRNA. The probes were labeled using a multiprime DNA-labeling system kit (Amersham) with [<sup>32</sup>P]dCTP. Relative densities of hybridization signals were determined by scanning the autoradiograms at 525 nm (Model CS-9000, Shimadzu) and normalized to the values of the 18S rRNA.

**Gel mobility shift assay** Nuclear extracts were prepared from rat livers as described by Gorski et al [25]. Male Wistar rats were fasted for 2 days and then refed a high-carbohydrate/fat-free diet for 5 h. The high-carbohydrate diet contained 67% sucrose. Antibody against Sp1 or Sp3 was added to the reaction mixture and incubated for 1 h at room temperature prior to adding the labeled probes as described previously [16]. The nucleotide sequences of IR(-618/-593), IR(-618/-593) mutants, IR(-603/-593), leptin(-101/-83), ACL(-64/-41), FAS(-57/-35) and Sp1 are shown above.

## Results and Discussion

**Effects of glucose and insulin on IR transcription** To explore possible regulatory elements in the 5'-flanking region of the IR gene, double-stranded oligonucleotides corresponding to -618 to -593 of the IR gene were synthesized and linked to the PGL3 promoter. The plasmid DNA was then transfected into rat hepatocytes. The cells were cultured in the presence of glucose with or without insulin for 48 h, and then were assayed for luciferase activity, as a reporter activity for IR transcription. As shown in Fig. 1, the reporter activity was significantly increased in the presence of glucose and more increased in the presence of glucose/insulin. The stimulation by glucose/insulin was reduced by 30% in arachidonic acid-treated hepatocytes and by 34% in leptin-treated hepatocytes. These results were similar to those found earlier for the transcriptions of the fatty acid synthase, FAS(-57/-35), ATP citrate-lyase, ACL(-64/-41) and leptin(-101/-83) genes, which promoter sequences have a high similarity to IR(-618/-593). However, the IR gene was less responsive to insulin stimulation and more responsive to glucose stimulation than those.

The IR(-618/-593) contains a Sp1 binding site (-601 to -593) and three overlapping Sp1 binding sites (-618 to -602). To test the binding of Sp1 or Sp3 to the DNA, rat hepatocytes were cotransfected with the IR(-618/-593) linked to the reporter gene and the Sp1 or Sp3 expression vector (Fig.2). The IR gene reporter activity was reduced in the presence of the Sp1 expression vector, whereas the activity was markedly increased in the presence of the Sp3 expression vector.

It is suggested that the Sp1 consensus sequence, located between nucleotides -618 and -593 of

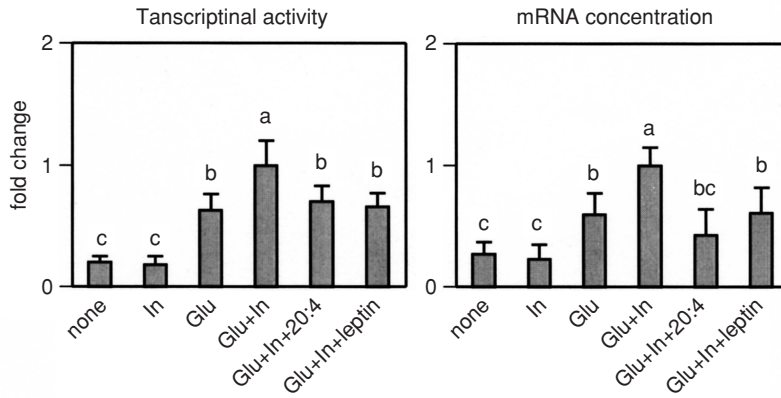


Fig. 1 Effects of glucose, insulin, PUFA and leptin on transcriptional activity and mRNA concentration of IR gene. The plasmid construct containing IR(-618/-593) linked to PGL3 promoter was introduced into primary cultured hepatocytes. The cells were cultured for 48 h to measure the transcriptional activities or for 24 h to measure the mRNA concentrations in William'E medium in the presence of 20 mM glucose(Glu) and/or 0.1 $\mu$ M insulin(In). When included, 0.1 mM arachidonic acid(20:4) or 0.3 $\mu$ M leptin was added. The transcriptional activities and endogenous mRNA concentrations of IR are normalized to Glu+In. Means with different superscript letters are significantly different at P<0.05. Mean  $\pm$  SD(n=4).

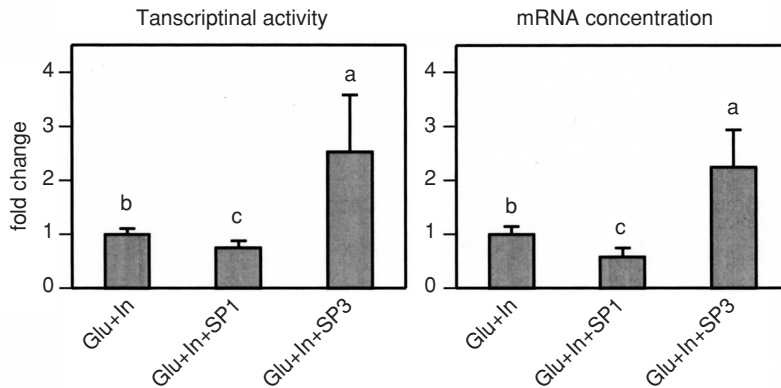


Fig. 2. Effects of Sp1 family on transcriptional activity and mRNA concentration of IR gene. IR(-618/-593) linked to PGL3 promoter was cotransfected to rat hepatocytes with Sp1 or Sp3 expression vector. The cells were cultured for 48 h to measure the transcriptional activities or for 24 h to measure the mRNA concentrations in William'E medium in the presence of 20 mM glucose(Glu) and 0.1 $\mu$ M insulin(In). The transcriptional activities and endogenous mRNA concentrations of IR are normalized to Glu+In. Means with different superscript letters are significantly different at P<0.05. Mean  $\pm$  SD(n=4).

the IR gene, can bind to Sp1 and/or Sp3, and that the bound Sp1 inactivated transcription and the bound Sp3 activated. These results were similar to those found earlier for the transcriptions of FAS(-57/-35), ACL(-64/-41) and leptin(-101/-83), which promoter sequences have a high similarity to IR(-618/-593).

To further define the sequence involved in the glucose/insulin responsiveness of the IR promoter, mutagenesis was performed in three overlapping Sp1 binding sites from -618 to -602 of the IR gene (M1, M2, M3) and in a Sp1 site from -601 to -593 (M4). Mutation of either one of the Sp1 binding sites (-618/-593) did not significantly affect the transcription. However, mutation of three (M1, M2, M3) or four (M1, M2, M3, M4) Sp1 binding sites resulted in a loss of responsiveness to glucose/insulin, as shown in Table 1. These results showed that lacking either one of the Sp1 binding sites did not have much affect on transcription.

**Table 1. Effects of mutation of the IR gene on transcriptional activity**

	Transcriptional activity (fold)			
	None	Insulin	Glucose	Insulin+glucose
IR(-618/-593)	0.20 ± 0.05 <sup>c</sup>	0.18 ± 0.07 <sup>c</sup>	0.67 ± 0.11 <sup>ab</sup>	1.0 ± 0.30 <sup>a</sup>
M 1	0.28 ± 0.09 <sup>bc</sup>	0.13 ± 0.03 <sup>d</sup>	0.66 ± 0.20 <sup>a</sup>	0.77 ± 0.22 <sup>a</sup>
M 2	0.36 ± 0.16 <sup>bc</sup>	0.39 ± 0.15 <sup>bc</sup>	0.74 ± 0.19 <sup>a</sup>	0.89 ± 0.18 <sup>a</sup>
M 3	0.27 ± 0.09 <sup>bc</sup>	0.34 ± 0.13 <sup>b</sup>	0.64 ± 0.19 <sup>a</sup>	0.95 ± 0.28 <sup>a</sup>
M 4	0.37 ± 0.09 <sup>bc</sup>	0.54 ± 0.13 <sup>ab</sup>	0.78 ± 0.19 <sup>a</sup>	0.95 ± 0.35 <sup>a</sup>
M1 ~ M4	0.38 ± 0.11 <sup>bc</sup>	0.31 ± 0.15 <sup>bc</sup>	0.30 ± 0.11 <sup>bc</sup>	0.340 ± 0.10 <sup>b</sup>
M1 ~ M3	0.40 ± 0.11 <sup>bc</sup>	0.42 ± 0.05 <sup>b</sup>	0.25 ± 0.11 <sup>c</sup>	0.26 ± 0.13 <sup>c</sup>



The wild type sequence from -618 to -593 of the IR promoter is shown, and Sp1 binding sites are indicated as boxes. M1- M4, mutants of IR gene, were shown and the sequences substituted are underlined.

Transcriptional activities were normalized to the level of the wild type sequence of IR(-618/-593) in the presence glucose/insulin. Means with different superscript letters in each item are significantly different at P<0.05. Mean±SD (n=4).

**Effects of glucose and insulin on IR mRNA concentrations** The effects of insulin, glucose, PUFA, Sp1 family and leptin on endogenous IR mRNA concentrations were measured after 24 h incubation of the hepatocytes. As shown in Fig. 1, the addition of glucose clearly enhanced the mRNA concentration of IR, and the mRNA induction was more increased by

addition of insulin. The glucose/insulin stimulation was completely suppressed by arachidonic acid (20:4) or leptin. The IR mRNA concentration was reduced in the presence of the Sp1 expression vector, and was increased in the presence of the Sp3 expression vector (Fig. 2).

These results similarly changed to the IR transcriptional activities. These support that the IR transcription stimulated by glucose/insulin was suppressed by Sp1 and stimulated by Sp3.

**Gel mobility shift assays** To identify and characterize potential protein-binding activity associated with the IR(-618/-593) region, an electrophoretic mobility shift assay was used.  $^{32}$ P-Labeled oligonucleotide IR(-618/-593) was incubated with nuclear extracts of rat liver and subjected to non-denaturing polyacrylamide gel electrophoresis. Three bands of DNA-protein complexes were observed and the formation of these complexes were competed against increasing amounts of unlabeled oligonucleotide IR(-618/-593), as shown in Fig. 3. The results demonstrated the specificity of the DNA-protein complex formation. Similarly, in the presence of excess unlabeled mutant M1, M2, M3 and M4 of IR gene, competitions with the IR(-618/-593) were observed. These suggest that sequences between -618 to -593 are essential for the binding of nuclear factor(s) to this putative glucose/insulin response region. Moreover, we tested whether the leptin, FAS and ACL sequences could compete for the nuclear factors binding to IR (-618/-593). ACL(-64/-41) and Sp1 competed away to all the three bands, whereas leptin (-101/-83) and FAS(-57/-35) competed for the band 2 and band 3 (Fig. 3).

When  $^{32}$ P-labeled oligonucleotide IR(-603/-593) was incubated with nuclear extracts of rat liver,

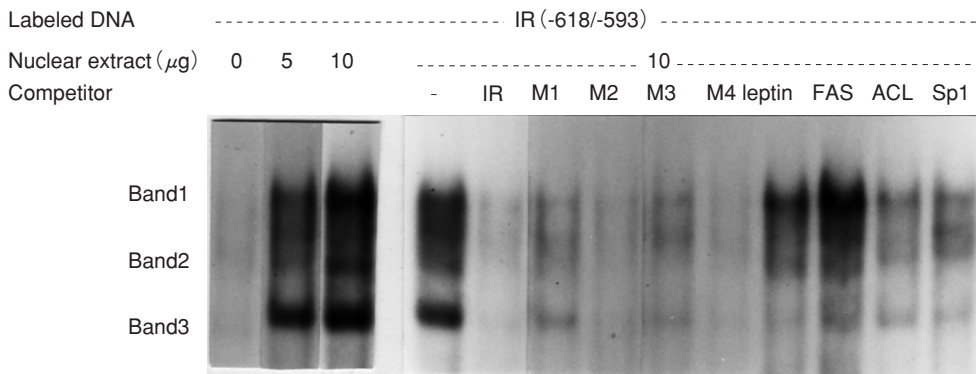


Fig. 3. Gel mobility shift assays using liver nuclear extracts with end-labeled IR(-618/-593). Increasing amounts of rat liver nuclear extracts were incubated in the presence of end-labeled oligonucleotide IR(-618/-593) and then loaded onto a 4% non-denaturing polyacrylamide gel. Competitor DNAs [IR(-618/-593), mutants of IR(-618/-593) : M1, M2, M3 and M4, leptin(-101/-83), FAS(-57/-35), ACL(-64/-41) or Sp1] were added in 30-fold molar excess relative to labeled DNA. The species of competitor DNAs are indicated at the top.

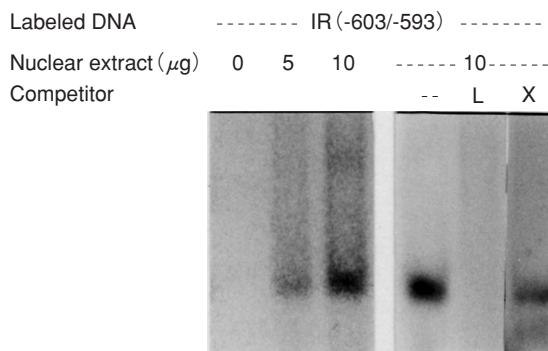


Fig. 4. Gel mobility shift assays using liver nuclear extracts with end-labeled IR(-603/-593). Increasing amounts of rat liver nuclear extracts were incubated in the presence of end-labeled oligonucleotide IR(-603/-593) and then loaded onto a 4% non-denaturing polyacrylamide gel. Competitor DNAs [L: IR(-618/-593), X: IR(-603/-593)] were added in 30-fold molar excess relative to labeled DNA. The species of competitor DNAs are indicated at the top.

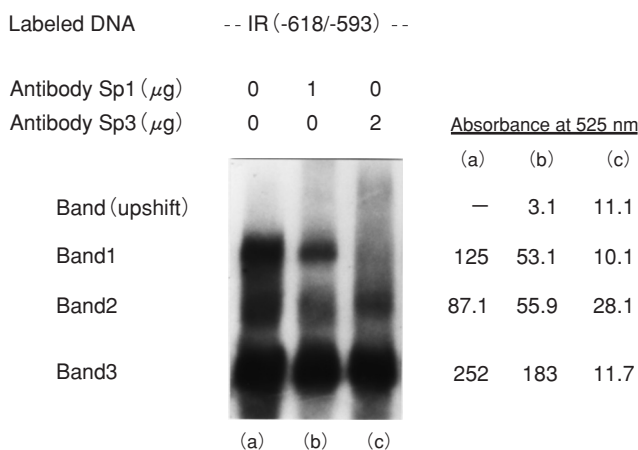


Fig.5. DNA mobility supershift assay. 5  $\mu\text{g}$  of nuclear extracts from rat liver were incubated with or without Sp1 and Sp3 antibodies (1  $\mu\text{g}$  or 2  $\mu\text{g}$ , respectively) before addition to end-labeled oligonucleotide IR(-618/-593) and then loaded onto a 4% non-denaturing polyacrylamide gel. To measure the DNA binding the exposed film was scanned at 525 nm and the optical densities are shown in the right of fluorograms.

the DNA-protein complexes were also observed and the formation of these complexes were competed against the unlabeled oligonucleotide (Fig. 4). The results in Fig. 3 and Fig. 4 demonstrated that sequences between -618 to -593 are essential for the binding of nuclear factor (s) to this putative glucose/insulin response region.

To demonstrate further that Sp1 family binds to the IR gene, specific antibody against Sp1 or Sp3 was added in the gel mobility shift assay (Fig. 5). In the presence of Sp1 antibody, the formation of three bands for the DNA-protein complexes, especially band 1 and band 2 were



efficiently inhibited. In the presence of Sp3 antibody, the formation of three bands was also inhibited. These results show that three bands represent specific IR(-618/-593)-containing protein-DNA complex (es) which contain Sp1 and Sp3.

Sp1 and Sp3 can bind to the sequences located between -101 and -83 of the leptin gene, and that the bound Sp1 inactivated the leptin transcription, whereas the effect of Sp3 was unclear [18]. Similarly, Sp1 and Sp3 bound to nucleotides -57 to -35 of the FAS gene and nucleotides -64 to -41 of the ACL gene, and the binding of Sp1 inactivated the transcription of the FAS(-57/-35) and ACL(-64/-41) genes whereas the binding of Sp3 stimulated the transcription of these genes [26]. Sp1 bound to nucleotides -618 to -593 of IR gene and the binding of Sp1 inactivated the transcription of the IR gene, and the binding of Sp3 increased the transcription of genes. These nucleotide regions have been postulated to be the response elements of glucose/insulin activation which are inactivated by PUFA, Sp1 or leptin. Thus, we postulated that Sp1 and Sp3 are somehow involved in the glucose/insulin activation and in the PUFA or leptin inactivation of the IR gene as well as the FAS, ACL and leptin genes, suggesting that these genes are relatively regulated.

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