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LOCALIZATION OF AN ADIPOCYTE-SPECIFIC RETINOIC ACID RESPONSE DOMAIN CONTROLLING S14 GENE TRANSCRIPTION

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SUMMARY: S14 gene transcription is induced by retinoic acid (RA) in cultured adipocytes, but not preadipocytes. Transfection of 3T3-L1 cells with S14-CAT fusion genes showed that adipocyte-specific RA responsive cis-acting elements are located between -1588 and -1381 bp upstream from the 5′ end of the S14 gene. This region has enhancer-like properties. In contrast, an artificial RA response element conferred RA control to a CAT gene in both preadipocyte and adipocyte phenotypes indicating that the RA regulatory network is functional in both phenotypes. These studies show that RA receptors interact with adipocyte-specific transcription factors to control S14 gene expression.

Recent studies indicate that retinoic acid (RA) acting through its nuclear receptors may play an important role in carbohydrate and lipid metabolism (1-5). We reported that RA acted alone and in combination with glucocorticoids to regulate S14 gene transcription in cultured adipocytes (1,2). The S14 protein (17 kDa, 4.9 pI) has served as a model for hormonal and nutrient control of lipogenic gene expression. While RA significantly induced S14 gene transcription in adipocytes, RA had no effect on S14 gene expression in preadipocytes. This observation indicated RA action on S14 gene expression required adipocyte-specific factors to function. In this study, a

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transfection approach was used: 1) to determine whether the RA regulatory network was functional in both preadipocyte and adipocyte phenotypes; and 2) to localized the RA responsive region controlling S14 gene transcription in adipocytes.

MATERIALS AND METHODS

Plasmid Constructions: The following plasmids were constructed to localize RA responsive cis-acting elements upstream from the 5′ end of the rat S14 gene. pCAT(An) and pS14-2.1-CAT were obtained from H. Towle (University of Minnesota; 6). pCAT(An) contains the bacterial chloramphenicol acetyltransferase (CAT) gene and 2 SV40 polyadenylation signals. pS14-2.1-CAT contains rat liver S14 genomic sequences extending from -2111 to +19 bp placed adjacent to the CAT gene. pS14-4.3-CAT contains S14 sequences extending from -4315 to +19 bp, and was constructed by inserting a 2.2 kb BamHI-Hind III S14 genomic fragment from pEMBLS14-13E (7) into pS14-2.1-CAT. The S14-1.6-CAT plasmid contains S14 sequences from -1601 to +19 bp, and was prepared by isolating the 1.6 kb Xho I fragment from pS14-2.1-CAT, and inserting this fragment into the Xho I site of pCAT(An). pS14-1.0-CAT contains S14 sequences from -1074 to +19 bp, and was constructed by inserting a 519 bp Pst I fragment into the Pst I site of pS14-.29-CAT. pS14-.29-CAT contains S14 sequences from -290 to +19 bp, and was constructed by digesting pS14-1.6-CAT with Pst I, isolating the vector, and re-ligating. pS14-.087-CAT contains sequences from -87 to -8 bp, and was constructed by placing the insert from pS14(-87 to -8) (11) into pCAT(An).

The following set of plasmid constructions were made to delineate further the RA responsive region. The region between -1588 and -1069 bp was excised by restriction of pS14-PA(0.5) with Pst I and the insert placed into the BamH I site of pS14-.29-CAT. The 519 bp insert was restricted with Ava I (-1386 bp). The fragment extending from -1588 to -1381 bp was cloned into the 5′ BamH I site of pS14-.087-CAT (reverse orientation), and into pS14-.29-CAT (correct orientation). The fragment extending from -1386 to -1069 bp was similarly cloned into pS14-.087-CAT. A control RARE was synthesized (5′GATCCTCAGGTCACCAGGAGGTCAGAG3′) corresponding to that described by Umesono et al. (9), and was cloned into the BamH I site of pS14-.087-CAT.

To determine if the S14 enhancer retained hormonal and tissue-specific control when adjacent to a heterologous promoter, the 519 bp PstI insert containing sequences from -1588 to -1069 bp was subcloned adjacent to a thymidine kinase (TK) promoter-CAT. In addition, S14 enhancer fragments -1588 to -1381 bp, and -1386 to -1069 bp were inserted into the BamH I site of pTK-CAT. pTK14A was obtained from R.J. Koenig (University of Michigan; 10). pTK14A contains a thyroid hormone response element that was excised by digestion with BamHI. The plasmid was purified and re-ligated to form pTK-CAT.

Culture and Transfection of 3T3-L1 Cells: 3T3-L1 cells were obtained from H.S. Sul (Harvard University), and were cultured and differentiated as described previously (11). 3T3-L1 fibroblasts were transfected with pSV2-NEO (2 μg; obtained from S. Conrad (Michigan State University)) in the presence of promoter/CAT expression plasmids (20 μg) using lipofection (12). Cell cultures were treated with RA only after preadipocytes were confluent or adipocytes were >85% differentiated. Cells were

treated with 1 μM RA for 72 h with 1 change of media after 48 h. Following hormone treatment, cell homogenates were analyzed for the presence of CAT (13, 14). Protein concentration of cell extracts were determined using the Bio-Rad Protein Assay (15) in order to normalize CAT assays for protein concentration. CAT activity in fibroblasts represents the mean of duplicate samples. Range between fibroblasts duplicates was <15%. CAT activity in adipocytes represents the mean of triplicate samples and the S.E.M. was <15%.

RESULTS AND DISCUSSION

Previous studies established that RA receptors (RARα) were present in preadipocytes and adipocytes (1,2). To determine whether these RARs were functional in both phenotypes, 3T3-L1 preadipocytes were stably transfected with a plasmid containing an artificial RARE (9) ligated upstream from the S14 minimal promoter-CAT fusion gene. RA induced CAT activity 6.2- and 10-fold from this construct in preadipocytes and adipocytes, respectively (Fig. 1), indicating that RARs were functional in both phenotypes.

We next determined whether S14 5′-flanking sequences directed adipocyte-specific RA regulation of S14 gene transcription. The region between -4315 and +19 bp was fused to

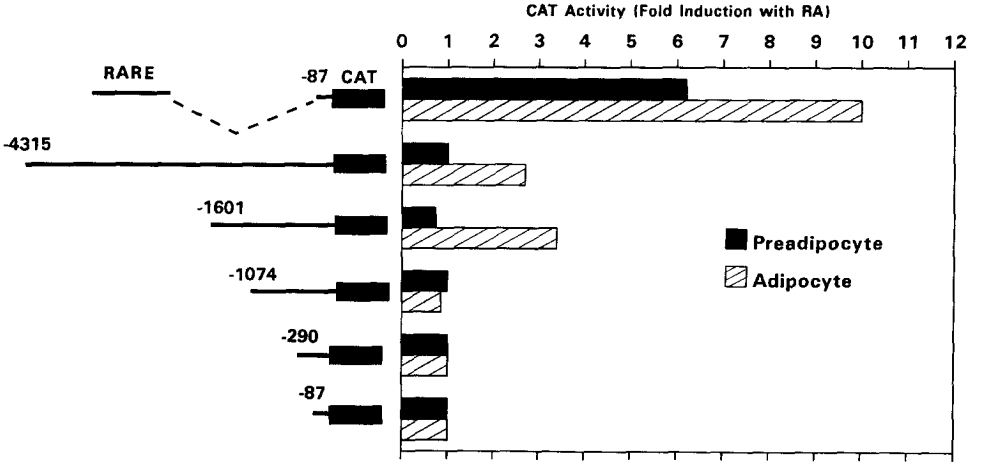

Figure 1. Adipocyte-specific control of S14-CAT. 3T3-L1 cells were stably transfected with RARE-CAT or S14-CAT constructs as indicated. Preadipocytes and adipocytes were treated with vehicle or 1 μM RA for 72 h. CAT activity was determined and data are expressed as fold induction in response to RA.

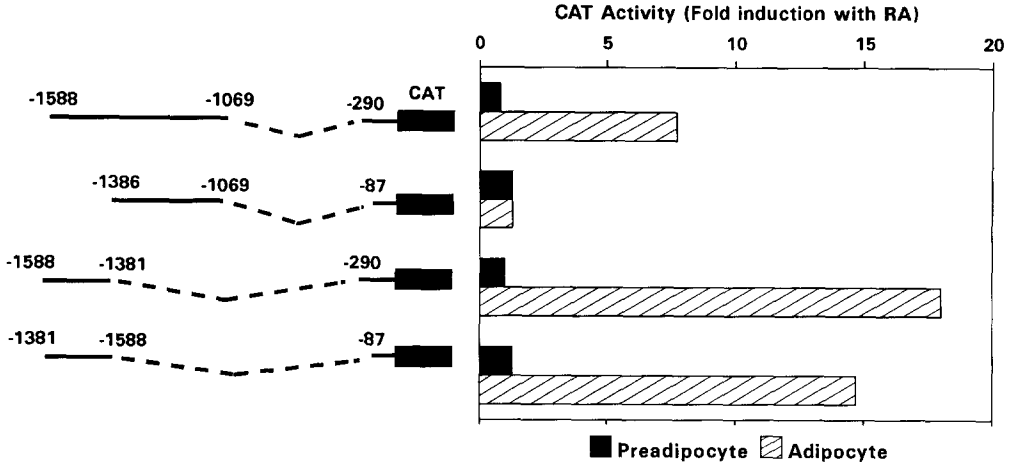

Figure 2. Localization of the S14 RA responsive region. S14 sequences, -1588 to -1069, -1386 to -1069, and -1588 to -1381 bp, were inserted adjacent to S14-CAT constructs containing basal S14 promoter sequences. Experiments were performed and data expressed as in Fig. 1.

the CAT reporter gene and stably transfected into 3T3-L1 cells. These sequences conferred RA control in the adipocyte phenotype, but not the fibroblast phenotype (Fig. 1). Deletion analysis showed that adipocyte-specific RA control was retained with 5′ end points at -1601 bp, but was lost with deletions shorter than -1074 bp.

The -1588 to -1069 bp region was further dissected by placing the -1588 to -1069 bp region adjacent to the -290 bp promoter element. RA induced CAT activity 7.7-fold in 3T3-L1 adipocytes, but not in preadipocytes (Fig. 2). Further subdivision of this region revealed that while sequences extending from -1388 to -1069 did not confer RA responsiveness in either 3T3-L1 phenotype, the region extending from -1588 to -1381 bp conferred a 14- to 20-fold induction in adipocytes, without providing RA regulation in fibroblasts. The -1588 to -1381 bp element was active in both orientations.

We next determined whether these elements function when ligated to the heterologous thymidine kinase (TK) promoter. Although the TK promoter alone did not support RA responsiveness

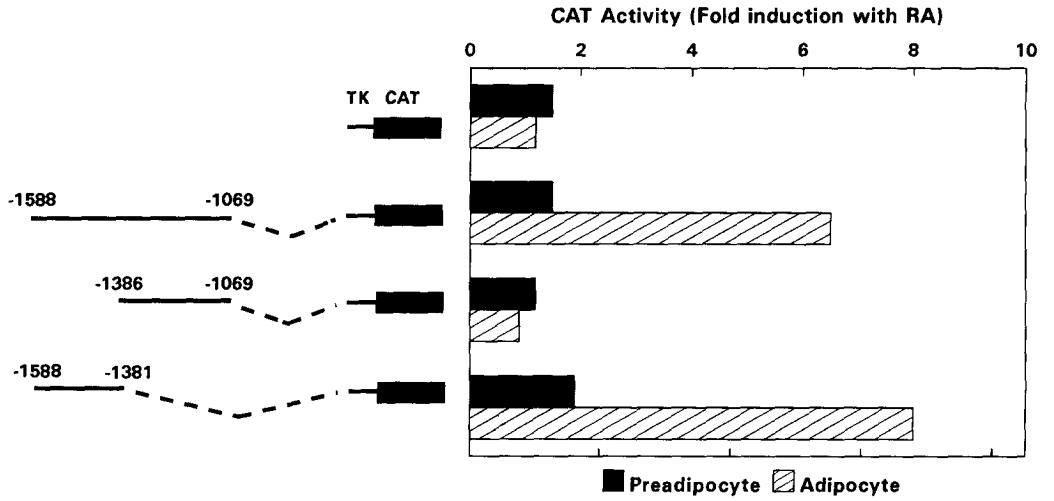

Figure 3. Adipocyte-specific RA induction of the thymidine kinase promoter. S14 sequences (-1588 to -1069, -1386 to -1069, and -1588 to -1381 bp) were inserted adjacent to the thymidine kinase promoter in TK-CAT and stably transfected into 3T3-L1 cells. Experiments were performed and data expressed as in Fig. 1.

(Fig. 3), placing S14 sequences -1588 to -1069 bp adjacent to TK-CAT conferred an adipocyte-specific 6-fold induction in CAT activity in response to RA. Inserting S14 sequences extending from -1388 to -1069 adjacent to TK-CAT did not confer RA control in either 3T3-L1 phenotype. However, inserting S14 sequences -1588 to -1381 bp caused a 8-fold induction in adipocytes, while no effect was observed in preadipocytes. These results indicate that the adipocyte-specific RA responsive region has enhancer-like properties, i.e. it works in an orientation-independent manner and functions with either homologous or heterologous promoters.

The nucleotide sequence for the S14 gene extending from -4315 to +494 has been described (7). The S14 RA enhancer region (-1588 to -1381 bp) was examined for similarity to known RA response elements. Many S14 sequences (>20) share weak identity with RARE's from β_1-laminin (16), RARβ1 (17) alcohol dehydrogenase (18), phosphoenolpyruvate carboxykinase (4,19), cellular retinol binding protein I genes (20), or the more

general RA response elements described by Leid et al. (21). This weak identity may indicate that putative S14 RA response element(s) binds RA receptors weakly or not at all. Binding of RA receptors may be facilitated or inhibited by binding of other transcription factors. Since RARα are present in both 3T3-L1 phenotypes (1,2), we hypothesize that binding and/or activity of RA receptors is modulated by other tissue-specific nuclear factors. Our studies do not rule out indirect effects of RA through the regulation of non-receptor transcription factors.

Finally, the RA responsive region identified for the S14 gene is located within a DNase I hypersensitive site (i.e. Hss-2; 7). This region also harbors response elements for glucocorticoid (2), tissue-specific (2), and insulin/carbohydrate regulatory factors (7,22). Detailed analysis of the cis- and trans-acting factors interacting with this region will provide important clues to how multiple signalling pathways converge to confer tissue-specific hormonal control to a specific gene.

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