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# IDENTIFICATION OF GLUCOCORTICOID- AND CYCLIC AMP- RESPONSIVE ELEMENTS OF THE RAT SERINE DEHYDRATASE GENE: DIFFERENCE IN RESPONSES OF THE TRANSFECTED AND CHROMOSOMAL GENES

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**SUMMARY**: Transcription of the gene coding for serine dehydratase (SDH, EC 4.2.1.13) in rat liver is induced 3-4 fold by glucocorticoids plus glucagon, but not by either hormone alone. For identification of the DNA elements mediating the glucocorticoid- and cyclic AMP-regulated expression of the SDH gene, primary cultures of adult rat hepatocytes were transfected with a fusion gene consisting of the 2.15 kb 5'-flanking sequence of the SDH gene linked to the coding sequence of the gene for chloramphenicol acetyltransferase (CAT). CAT assay demonstrated that transient expression of the SDH-CAT fusion gene was inducible by either dexamethasone or dibutyryl cyclic AMP, but that the effects of these inducers were not additive or synergistic. These results suggest that some structural organization of the DNA influences the hormonal actions in regulation of gene expression. 

Serine dehydratase (SDH) catalyzes the conversion of serine to pyruvate in hepatic gluconeogenesis. The enzyme is expressed mainly in the liver (1) and its activity appears in the suckling period (1,2). SDH is subject to positive and negative regulations by hormones, and so is a good model for use in studies on multihormonal regulation of specific genes (3). Gluconeogenesis and ureogenesis have long been known to be induced synergistically by glucocorticoids and glucagon, and these effects have been called "permissive action" of glucocorticoids, but the molecular mechanisms of this action are unknown. Induction of SDH is a typical example of "permissive effect" since neither hormone alone can induce the enzyme, unlike in the cases of other gluconeogenic enzymes (4-7). In primary cultures of adult rat hepatocytes, both hormones cooperate to increase transcription of the SDH gene and this causes increase in the level of $mRNA^{SDH}$ and enzyme synthesis (3). Conversely, insulin and epinephrine ($\alpha_1$ action) inhibit transcription of the SDH gene (3).

Abbreviations used:
SDH, serine dehydratase; CAT, chloramphenicol acetyltransferase; cAMP, adenosine 3', 5'-cyclic monophosphate; $bt_2cAMP$, $N^6$,2'-dibutyryl adenosine 3', 5'-cyclic monophosphate; GRE, glucoccorticoid responsive element; CRE, cAMP responsive element.

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In general, glucocorticoids and glucagon [or hormones increasing the intracellular cyclic AMP (cAMP) level] stimulate transcription through specific sequences in target genes that are called glucocorticoid-responsive element (GRE) and cAMP responsive element (CRE), respectively.

A genomic clone encoding rat SDH has been isolated, and sequences homologous to the consensus GRE and CRE have been found in the 5'-flanking sequence of this SDH gene (8,9). In this study, we examined the expression in primary cultures of adult rat hepatocytes of the SDH-chloramphenicol acetyltransferase (CAT). By CAT assay, the GRE and CRE were identified 2,145 bp upstream of the cap site. Unexpectedly, transient expression of the fused gene was induced by either the synthetic glucocorticoid dexamethasone or dibutyryl cAMP ($bt_2$cAMP) and the two compounds did not have synergistic effects.

## MATERIALS AND METHODS

Materials: T4 DNA ligase was obtained from New England Biolabs or Takara Shuzo. T4 DNA polymerase was obtained from Takara Shuzo. Restriction endonucleases were from Takara Shuzo, Toyobo, and New England Biolabs, Inc. Bal31, pUC19 plasmid vector and *Hin*dIII linker were from Toyobo. Acetyl CoA was from Sigma. Williams' medium E, Dulbecco modified MEM, new born calf serum, and fetal calf serum were from Flow Laboratories. [$^{14}$C]Chloramphenicol (57 mCi/mmol) was obtained from Amersham Japan.

Construction of SDH-CAT Chimeric Recombinant and SDH-CAT 5'-Deletion Mutants: A 2.95 Kb *Eco*RI fragment of the SDH gene (9) containing 2.15 kb of the 5'-flanking sequence and 0.80 kb downstream of the cap site was subcloned into pUC19. The plasmid was linearized with *Ssp*I whose site was located inside the vector, and digested with Bal31. The ends were blunted with T4 DNA polymerase, and *Hin*dIII linkers were ligated onto the linearized molecule. A 2.30 kb *Eco*RI-*Hin*dIII fragment consisting of the sequence from residue -2145 to +157 relative to the cap site of the SDH gene was inserted into the *Eco*RI-*Hin*dIII sites of pKSCAT, [originally derived from pSV2CAT and was kindly provided by Dr. Koichi Suzuki (10)]. The chimeric recombinant, 2145SDH-CAT, was digested with appropriate restriction endonucleases to produce 5'-deletion mutants. The resulting fragments containing -2004/+157 (*Pvu*II/*Hin*dIII), -765/+157 (*Kpn*I/*Hin*dIII), and -253/+157 (*Sac*I/*Hin*dIII) linked to pKSCAT were self-ligated. These 5'-deletion mutants were named 2004SDH-CAT, 765SDH-CAT, and 253SDH-CAT, respectively.

Cell Culture and DNA Transfection: Parenchymal hepatocytes were isolated from adult male rats (150-200 g) of the Wistar strain by perfusion of the liver *in situ* with collagenase (3). The cells were plated at 1 x $10^5$ cells/cm$^2$ in 6 cm-plastic dishes and cultured in Williams' medium E containing 5 % new born calf serum, $10^{-9}$ M insulin and $10^{-9}$ M dexamethasone under 5 % $CO_2$ in air for 6 hours. Then DNA transfection was carried out by the calcium phosphate method as described previously (11) with minor modifications. Plasmid DNAs for transfection were prepared by the alkali/SDS method and banded by ultracentrifugation on a cesium chloride/ethidium bromide equilibrium density gradient by the standard technique (12). Samples of 15 μg of supercoiled DNA per dish were transfected. pSV2CAT and pKSCAT were used as positive and negative controls, respectively. After 14 hours, DNA was removed by replacing the medium by hormone-free Williams' E supplemented with 5 % new born calf serum. Then, to increase the transient expression of the transfected DNA, the cells were treated with 5 mM sodium butyrate (13) for 24 hours, and harvested 48 hours after the butyrate-treatment. When cells were treated with the hormones, either $10^{-7}$ M dexamethasone or 5 x $10^{-5}$ M $bt_2$cAMP, or both were added 24 hours before harvesting. A human hepatoblastoma cell line (HepG2) and a rat hepatoma cell line (H4IIE) were grown in Dulbecco modified MEM supplemented with 10 % fetal calf serum. These cells were transfected by either electroporation in a Gene Pulser™ (BIO-RAD) or the calcium phosphate method.

CAT Assays: Cells were harvested 62 hours after transfecton and disrupted by freeze-thawing. CAT assays were performed as described by Kingston (14) using equal amounts of cell extract in

terms of protein, but cell extracts were preheated at 60 °C for 10 min (15). Quantitative results were obtained by cutting out the areas of the chromatogram corresponding to the acetylated forms of [$^{14}$C]chloramphenicol and counting their radioactivity in a liquid scintillation counter.

## RESULTS AND DISCUSSION

Expression of the Rat SDH-CAT Fusion Gene in Primary Cultures of Hepatocytes: To identify sequences mediating induction of the SDH gene by glucocorticoids and cAMP, we constructed a fusion gene (2145SDH-CAT) consisting of the SDH 5'-flanking sequence (residue -2145 to +157) fused upstream of the CAT gene. Expression of this plasmid was studied after its transient introduction into a rat hepatoma cell line (H4IIE), a human hepatoblastoma cell line (HepG2), and primary cultures of adult rat hepatocytes. We tried several procedures for transfecting primary cultures with DNA, including the use of calcium phosphate, liposomes, DEAE-dextran, and polybrene, and electroporation. Of these methods calcium phosphate coprecipitation and liposome-mediated transfection were the most efficient (data not shown). In the presence of dexamethasone and $bt_2$cAMP, primary cultures expressed the SDH-CAT fusion gene slightly (Fig. 1a, lane 6), but its expression in H4IIE and HepG2 cells was not detectable (Fig. 1a, lanes 2 and 4 ). pSV2CAT, used as a positive control of transfection, was expressed in HepG2 cells and in H4IIE at as high level as in primary cultures of hepatocytes (Fig. 1a, lanes 1, 3, and 5). These established cell lines did not express endogenous SDH even when cultured in the presence of dexamethasone and $bt_2$cAMP, as judged by measurement of enzyme activity and Northern blot

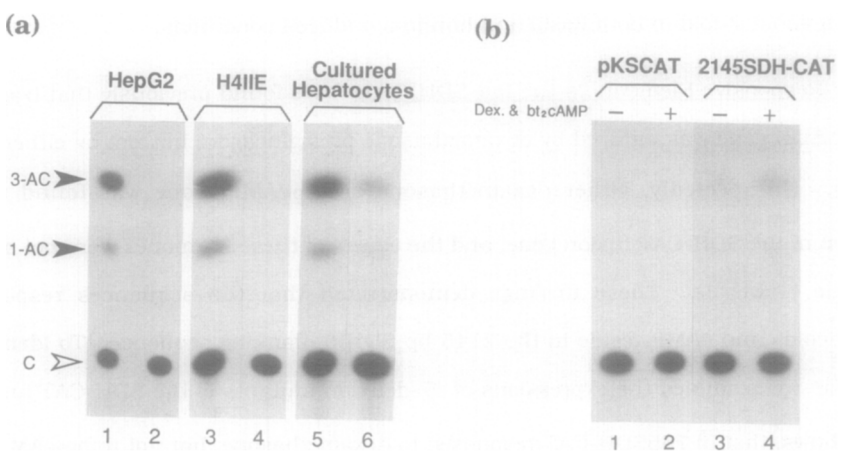


**Figure 1.** Expression of the SDH-CAT fusion gene in primary cultures of adult rat hepatocytes, but not in HepG2 or H4IIE cells. (a) HepG2, H4IIE cells or cultured hepatocytes were transfected with plasmid pSV2CAT (lanes 1, 3 and 5) or plasmid 2145SDH-CAT (lanes 2, 4 and 6) and CAT activity was determined in the transfected cells treated with $10^{-7}$ M dexamethasone and 5 x $10^{-5}$ M $bt_2$cAMP for 24 h before harvesting. The positions of chloramphenicol (C), and 1- and 3-acetylated chloramphenicol (1-AC and 3-AC) on TLC plates are shown by arrowheads. (b) Cultured hepatocytes were transfected with either plasmid pKSCAT or plasmid 2145SDH-CAT. Then, the cells were incubated without (-) or with (+) $10^{-7}$ M dexamethasone plus 5 x $10^{-5}$ M $bt_2$cAMP.

**Table 1.** Transient expressions of SDH-CAT fusion genes in primary cultures of adult rat hepatocytes. The radioactivity of acetylated forms of [$^{14}$C]chloramphenicol was counted in a liquid scintillation counter. Values are means for at least three independent experiments and are expressed relative to the basal value for 2145SDH-CAT.

| Addition | 2145SDH-CAT | 765SDH-CAT | 253SDH-CAT |
|---|---|---|---|
| None | 1.00 | 1.85 | 0.45 |
| Dexamethasone (Dex) | 1.73 | 3.23 | 0.39 |
| $Bt_2cAMP$ | 1.86 | 2.00 | 0.43 |
| Dex + $Bt_2cAMP$ | 1.75 | 2.63 | 0.38 |

analysis (data not shown). The absence of expressions of the endogenous SDH and the SDH-CAT fusion genes in these cells may have been due to loss of a positive transcription factor(s) or presence of a negative transcription factor(s).

Expression of the SDH-CAT fusion gene was increased 2- to 3-fold by treatment with dexamethasone and $bt_2cAMP$ (Fig. 1b, lanes 3 and 4). The pKSCAT plasmid, used as a negative control, was not expressed appreciably with or without the inducers (Fig. 1b, lanes 1 and 2). The induction of expression of the fusion gene in primary cultures was sequence-specific, because dexamethasone plus $bt_2cAMP$ did not affect the expression of pSV2CAT, or that of the β-galactosidase gene (pCH110) cotransfected with the SDH-CAT fusion gene (data not shown). As the SDH promoter activity seemed to be very low, the hepatocytes were treated with sodium butyrate to stimulate the promoter, as described previously (13). This treatment enhanced gene expression about 2-fold in both basal and hormone-induced conditions.

Hormone-Responsive Elements in the Rat SDH Gene: We found previously that transcription of the rat SDH gene was induced by dexamethasone plus glucagon, but not by either hormone alone (3). Unexpectedly, either dexamethasone or $bt_2cAMP$ alone was found to induce expression of the SDH-CAT fusion gene, and the effects of these hormones were not additive or synergistic (Table 1). These findings demonstrated that the sequences responding to glucocorticoids and cAMP reside in the 2145 bp SDH 5'-flanking sequence. To identify these sequences, we examined the expressions of 5'-deletion mutants of the SDH-CAT fusion gene. Table 1 shows, that (i) 765SDH-CAT responsed to dexamethasone, but not to $bt_2cAMP$, (ii) the basal expression of 765SDH-CAT was twice that of 2145SDH-CAT, and (iii) expression by 253SDH-CAT was less than half that of the parental plasmid in the absence of the hormones, and was not affected by either $bt_2cAMP$ or dexamethasone. The higher basal expression by 765SDH-CAT than by the parental plasmid indicated the presence of inhibitory sequences

| | | |
|---|---|---|
| GRE consensus | | **GGTACANNNTGTTCT** |
| GRE-like sequences | -2098 | TGAG**CA**AGC**TGTTCT** -2084 |
| in the rat SDH Gene | -411 | **AGTCCA**GGG**TGTTCT** -397 |
| | -388 | **AG**CTTCTAT**TGTTCT** -374 |
| CRE consensus | | **TGACGTCA** |
| CRE-like sequences | -1129 | **TGAAGTCA** -1122 |
| in the rat SDH Gene | -954 | **TGACG** -950 ----- |
| | ----- -574 | **CGTCA** -570 |

**Figure 2.** Sequences homologous with GRE and CRE consensus sequences in the rat SDH gene. The consensus sequence of GRE is cited from Refs. 7, 17 and 18, and that of CRE from Refs. 7 and 22.

between residue -2145 and -766. The low expression by 253SDH-CAT suggested that not only CAT- and TATA-like sequences (9), but also other sequences between residue -765 and -254 are concerned with the basal expression of the SDH gene.

The results of CAT assay are compatible with the findings of GRE-like sequences at -2098/-2084, -411/-397, and -388/-374, and of a CRE-like sequence at -1129/-1122 and two half motifs (TGACG) of CRE with inverse orientations at -954/-950 and -574/-570 in the 5'-flanking sequence of the SDH gene (Fig. 2) (9). Multiple GREs have been demonstrated in the 5'-flanking sequences of the tryptophan 2,3-dioxygenase (16), tyrosine aminotransferase (17), and phosphoenolpyruvate carboxykinase (7,18) genes, and these multiple GREs have been shown to function synergistically in stimulating transcription. Of the three putative GREs in the 5'-flanking sequence of the SDH gene, the two proximal sequences at -411/-397 and -388/-374 are probably functional GREs, because 2004SDH-CAT still retained ability to respond to glucocorticoids (data not shown).

In addition to apparent interaction of glucocorticoid receptor with the GRE(s) recent studies have suggested the importance of functional interactions between receptor molecules and other transcription factors, such as NF1, SP1, OTF, and the CACCC box-binding protein (18,19). Therefore, the putative GREs may not be the minimal DNA sequences required for glucocorticoid induction of the SDH gene.

Glucagon or cAMP analogues can stimulate transcription of the phosphoenolpyruvate carboxykinase gene and tyrosine aminotransferase (6,7) and can enhance glucocorticoid-stimulated transcriptions of the tryptophan 2,3-dioxygenase (4). In the phosphoenolpyruvate carboxykinase gene, CRE located between -100 and -82 is essential for both basal promoter activity and cAMP-regulated expression (7,20). However, CREs have not been identified for the tryptophan 2,3-dioxygenase and tyrosine aminotransferase genes.

There is no exact consensus sequence TGACGTCA in the 5'-flanking sequence of the SDH gene: the most closely homologous sequence, TGAAGTCA, is located between -1129 and -1122, but the CG doublet in the middle of the consensus sequence of CRE, which is conserved in most cAMP-responsive genes, is replaced by AG. Thus, it is more likely that the two half motifs at -954/-950 and -574/-570 are essential for binding some CRE-binding protein that functions as a dimer (21,22).

The results of CAT assay and deletion studies on the 5'-flanking sequence of the SDH gene suggest that the GRE(s) is located at least between -765 and -254 and that the CRE(s) is between -2004 and -765. However, the transient expression of the SDH-CAT fusion gene did not show synergistic responses to the two hormones. Possibly some sequence further upstream than -2145 is necessary for expression of the synergistic effects of these hormones. Another possibility is that the organization of chromosomal DNA differs from that of transfected DNA, and that the different responses are causes by differences in DNA topology. Even for transient expression, hormonal induction of transfected genes has been shown to depend on DNA topology (23). Therefore, stable transfection may be helpful in analyzing the mechanisms of the synergistic effects of glucocorticoids and cAMP.

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