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THE UPSTREAM PROMOTER OF THE HUMAN LDL RECEPTOR GENE DOES NOT CONTAIN A CYCLIC AMP RESPONSE ELEMENT

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Fusion genes containing segments of the promoter region of the human LDL receptor gene and the coding sequence of the bacterial enzyme, chloramphenicol acetyltransferase (CAT), were introduced into JEG-3 human choriocarcinoma cells. Constructs containing 177 base pairs of 5'-flanking DNA (pLDLR-CAT 234) or 6500 base pairs (pLDLR-CAT 6500) promoted CAT activity in transient expression assays. Although both pLDLR-CAT 234 and pLDLR-CAT 6500 contain sequences related to the recently reported consensus sequence for cyclic AMP reponsiveness, treatment of the transfected JEG-3 cells with 8-bromo-cAMP did not increase CAT activity. The cyclic AMP analog did, however, stimulate steroidogenesis and hCG secretion and increase CAT activity in cells transfected with p18X2SV1CAT, which contains two copies of an 18 base pair sequence corresponding to the cAMP-responsive element of the human α chorionic gonadotropin gene.

Low density lipoprotein (LDL) carried cholesterol is utilized as a hormone precursor by various steroidogenic cells (1). LDL is taken into these cells by a receptor-mediated process and we have previously reported that tropic stimulation of human granulosa cells leads to increased LDL receptor activity as a consequence of increased receptor synthesis which is driven by accumulation of the LDL receptor mRNA (2, 3). The mechanism by which gonadotropins, acting via their cyclic AMP second messenger, increase LDL receptor mRNA appears to involve both a reduction in sterol negative feedback of LDL receptor gene expression and

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Abbreviations used are: LDL, low density lipoprotein; CAT, chloramphenicol acetyltransferase; hCG, human chorionic gonadotropin.

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another process which may be independent of sterol negative feedback. Thus, cyclic AMP analogs increase LDL receptor mRNA levels in human granulosa cells to some extent even when the cells are provided with a potent sterol negative feedback signal (3). In contrast, 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA, which also increases in granulosa cells stimulated with cyclic AMP analogs, does not rise substantially when the cyclic nucleotide is added in the presence of sterol negative feedback (4). Cyclic AMP might increase LDL receptor mRNA by stimulating transcription of the LDL receptor gene. A consensus sequence (TGACGTCA) has been identified in the 5' flank of genes transcriptionally controlled by the cyclic nucleotide (5, 6). The promoter region of the LDL receptor gene contains sequences related to this consensus sequence, raising the possibility that they could be cis elements which serve as transcriptional enhancers (7). We examined the effects of 8-bromo-cyclic AMP on the expression of fusion genes comprised of various lengths of 5' flanking DNA from the human LDL receptor gene coupled to the bacterial enzyme chloramphenicol acetyltransferase (CAT) to explore this possibility. These fusion genes were introduced into progesterone-secreting JEG-3 human choriocarcinoma cells and promoter activity was assessed by transient expression of the CAT gene.

MATERIALS AND METHODS

Cells

JEG-3 choriocarcinoma cells were obtained from the American Type Culture Collection (Rockville, MD).

Fusion genes

Two different LDL receptor promoter-CAT fusion genes cloned into pSVO-CAT were utilized (8). The LDL receptor sequences terminated at the same 3' position within the transcribed region of the gene (-58) with the 5' end extending to -6500 or -234 bases upstream. These constructs were generously provided by Dr. David Russell of the University of Texas of Dallas. pSV2-CAT was provided by Dr. James Alwine (University of Pennsylvania). p18X2SVICAT was generously supplied by Dr. John Nilson (Case Western Reserve University).

Transient expression of fusion genes

Cells were transfected with plasmid DNA by the calcium phosphate coprecipitation technique (9). Briefly, cells were plated at a density of 5 x 105 per 6 cm culture plate in Dulbecco's minimum essential medium containing 25mM glucose, 4mM L-glutamine, 50 µg/ml gentamicin, 25mM HEPES, and 10% (vol/vol) fetal bovine serum 24h prior to transfection. In some experiments

JEG-3 cells received a DMSO "shock" to increase the efficiency of plasmid uptake. Medium was replaced with serum-free medium 24h after transfection. Some cultures were exposed to 1.5 mM 8-bromo-cAMP during the 24 h prior to harvest.

CAT assays

Cells were harvested with a plastic spatula, washed and then suspended in buffer consisting of 20mM Tris-HCl, pH 7.5, 10mM dithiotreitol, 15% glycerol. The cells were then subjected to 4-freeze-thaw cycles and the particulates sedimented by centrifugation. Aliquots of the supernatant solution were collected for protein assay by the method of Bradford (10) and for assay of CAT by the method of Gorman et al. (11). Incubations were carried out for 2 h, during which time product formation occured at a linear rate. The reaction products were extracted with ethyl acetate and separated by thin-layer chromatography (choloroform-methanol: 95: 5) and the acetylated chloramphenicol detected by autoradiography. The radioactivity in the products and remaining substrate was quantitated by liquid scintillation counting. Results are expressed as % conversion/100 ug protein/2 h.

Analytical Methods

hCG was quantitated using hCG MATA clone reagents (Serono, Norwell, MA). Progesterone was assayed using a solid phase system (Diagnostic Products Corp., Los Angeles, CA).

RESULTS

When the two fusion gene constructs, one containing 6.5 kb of 5' flanking DNA from the human LDL receptor gene (pLDLR-CAT 6500), the other containing 177 base pairs (pLDLR-CAT 234) were introduced into JEG-3 cells significant CAT activity was detected (Table 1). Little CAT activity was expressed when cells were transfected with pSV0CAT, the promoterless plasmid into which the LDL receptor gene sequences were inserted. These findings confirm the report of Sudhof et al. (8) which revealed that positive control elements are present within the initial 177 base pairs upstream of LDL receptor gene 5'-flanking DNA. 8-bromo-cAMP (1.5 mM) did not stimulate CAT activity in cells transfected with either pLDLR-CAT 234 or pLDLR-CAT 6500 (Table 1). However, the cyclic nucleotide analog did increase progesterone secretion 2.4-fold and increased hCG secretion 6-fold. As an additional control, we transfected the JEG-3 cells with p18X2SV1CAT, which contains two copies, in opposite orientation, of an 18 base pair sequence corresponding to the cAMP response element of the human α hCG gene. Treatment of the cells with 1.5 mM 8-bromo-cAMP following transfection with p18X2SV1CAT resulted in a significant

TABLE 1

Effects of 8-bromo-cAMP on Expression of CAT Activity

Plasmid	N	Basal CAT Activity % Conversion/100 ug protein/2 h	Induction Ratio
pSV0CAT	2	0.02	1.1
pLDLR-CAT 6500	4	6.2 ± 1.1	0.8 ± 0.2
pLDLR-CAT 234	3	7.3 ± 1.0	0.9 ± 0.3
p18X2SV1CAT	4	0.45 ± .15	6.0 ± 1.8
pSV2CAT	2	27.5	0.8

The induction ratio is the ratio of CAT activity observed in the presence of 8-bromo-cAMP to that expressed by cells in the basal state. N is the number of separate experiments performed. Values are the means ± S.E. for 3 or more experiments or the means from duplicate studies.

increase (6-fold) in CAT activity. 8-bromo-cAMP did not, however, affect CAT activity expressed by cells transfected with pSV2CAT.

DISCUSSION

The present studies reveal that a 177 base pair fragment of 5'-flanking DNA of the LDL receptor gene extending from -58 to -234 is sufficient for expression of the gene in a human steroidogenic cell. These findings confirm the previous work of Sudhof et al. (8) who utilized Chinese hamster ovary cells for most of their studies. The 177 base pair sequence contains two eight base pair sequences (-178 to -171: TGAGGTCA and -101 to-94: TGACGTGG) which are related to the recently identified consensus sequence of the cyclic AMP response element (TGACGTCA). We wished to know if cyclic AMP can stimulate transcription of the fusion genes, since the presence of cyclic AMP response elements in the 5' flank of the human LDL receptor gene could explain accumulation of LDL receptor mRNA which occurs in human granulosa cells challenged with hCG or 8-bromo-cAMP, even in the presence of sterol negative feedback (3, 4). However, we could not detect a stimulatory effect of the cyclic AMP analog on CAT expression in transient expression assays following introduction of either pLDLR-CAT 234 or pLDLR-CAT 6500. Preliminary studies

with mouse Y-1 adrenal tumor cells have also failed to reveal an effect of 8-bromo-cAMP with stably transformed cells as well as in transient expression assays. In contrast, 8-bromo-cAMP stimulated CAT activity in JEG-3 cells transfected with p18XSV1CAT, which contains the cyclic AMP response elements derived from the human α hCG 5' flanking DNA. Thus, our findings are inconsistent with the simple notion that a cyclic AMP response element similar to that described in the promoter regions of other genes controls the stimulation of LDL receptor expression in response to tropic hormones.

Transcription of the LDL receptor gene is known to be inhibited by sterol negative feedback and Sudhof et al. (8, 14) have reported that the elements of the LDL receptor gene which are required for the negative feedback response lie within the 177 base pairs which contain the positive control elements. The failure to detect cyclic AMP-responsiveness using pLDLR-CAT 234 or pLDLR-CAT 6500 could mean that cyclic AMP only stimulates transcription of the intact gene or that the cyclic AMP responsiveness is cell specific (i.e., only in human granulosa cells). While we cannot rule out the former possibility, the latter seems unlikely since trophoblastic cells express LDL receptors (12, 13). Alternatively, the effects of tropic stimulation on LDL receptor mRNA in human granulosa cells, which we have previously described, might be accounted for by alterations in the positive transcriptional factors or the negative feedback mechanism such that the factor or factors which suppress transcription of the LDL receptor gene are less efficient.

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