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Regulation of Ferredoxin Gene in Steroidogenic and Nonsteroidogenic Cells

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Ferredoxin is an electron transport intermediate for all the mitochondrial cytochromes *P*450. It is especially abundant in steroidogenic organs where it functions in steroid biosynthesis. The regulation of ferredoxin gene expression was studied in both steroidogenic and nonsteroidogenic cell lines. In steroidogenic cell line Y1, the expression of ferredoxin was stimulated by cAMP and repressed slightly by angiotensin II and phorbol ester PMA. These drugs exhibited the same effect on the basal promoter of the ferredoxin gene, which includes one TATA box and an SP1 site. In human adrenocortical cell line H295, the stimulation of the ferredoxin gene by cAMP was blocked by cycloheximide, as observed in bovine adrenocortical cell culture. In nonsteroidogenic cell lines such as HeLa and COS-1, the stimulation of ferredoxin gene expression by cAMP was not observed, although basal expression was strong. Transfection studies showed that the ferredoxin promoter could not be stimulated by cAMP in nonsteroidogenic cells. Therefore the steroidogenic cell-specific regulation and the general expression pattern appears to be a property unique to the ferredoxin gene.

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INTRODUCTION

Ferredoxin is a member of the electron transport chain in the mitochondria. Electrons are transported from NADPH, via ferredoxin reductase and ferredoxin, to terminal cytochrome *P*450, which use electrons and oxygen in a monooxygenase reaction to add a hydroxyl group to their substrates. Therefore, ferredoxin is important for the metabolism of substrates for mitochrondrial cytochrome *P*450.

Ferredoxin is found most abundantly in the adrenal cortex, where steroids are synthesized, therefore it is also called adrenodoxin. It is also found in the liver and kidney, for the metabolism of vitamin D and bile acid. Hence the name of hepatoredoxin and renodoxin. But sequencing of the cDNA showed that there is only a single protein species for all these proteins found in all these tissues, therefore ferredoxin may be a more appropriate term for this protein [1]. In addition to these tissues, ferredoxin mRNA was also found in every tissue at low levels [2].

The ferredoxin gene contains four exons and three introns [1, 3]. It is located on human chromosome 11 with two pseudogenes at chromosomes 20 and 21 [4]. Multiple polyadenylation sites are used resulting in mRNA species of different sizes. Since its major function is the participation of steroid synthesis in the adrenal cortex, its regulation pattern follows that of steroidogenic cytochrome *P*450. In bovine adrenal cortical cells [5], mouse adrenal tumor cell line Y1 [6], and human choriocarcinoma cell line JEG-3 [2], expression of ferredoxin mRNA is stimulated by cAMP in a delayed manner. It takes at least 6 h before this increase of ferredoxin mRNA is apparent.

The stimulation of steroidogenic cytochrome *P*450 is inhibited by cycloheximide [5], indicating that the synthesis of a labile intermediate protein may be required. Ablation by cycloheximide of cAMP stimulation of ferredoxin expression was observed only in the bovine adrenal cortical cell culture [5], but not in granulosa cells [7], Y1 [6], or JEG-3 cells [2]. This discrepancy may reflect an inherent difference of patterns of regulation in cells originated from different

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tissues or species. But more likely, it indicates that regulation of ferredoxin expression may be altered after cells were cultured.

Besides the work mentioned above, regulation of ferredoxin gene in nonsteroidogenic tissues, or by other reagents, has never been studied. This report presents our work on the study of ferredoxin gene regulation in both steroidogenic and nonsteroidogenic cells. We showed that in Y1 cells, cAMP was the major stimulator, while calcium slightly represses expression. We also demonstrated that the major *cis*-acting regulatory element of the ferredoxin gene is at the basal promoter, in the proximity of the TATA box. In addition, we found that in nonsteroidogenic cells, ferredoxin mRNA levels were not regulated by cAMP, reflecting the nature of the cell type-specific promoter.

EXPERIMENTAL

Cell culture

Y1, COS-1, I-10, H295, or HeLa cells were grown to near confluence before 100 μM phorbol ester PMA, 10 mM angiotensin II, 10 μM A23187, 50 μg/ml cycloheximide, 1 mM 8-Br-cAMP, or 0.1 μM dexamethasone was added to the culture and incubated for the amount of time indicated in each figure legend. Plasmids were transfected into cells using the calcium phosphate precipitation method as described earlier [8]. pWT-OVEC and OVEC have been described [8, 9].

Analysis of RNA

Northern-blot analysis was performed as described earlier [6]. The probes of human ferredoxin cDNA and GAP (glyceraldehyde-3-phosphate dehydrogenase) cDNA have been published [2]. After transfection, RNA was analyzed by primer extension as described earlier [6].

RESULTS

Regulation of ferredoxin in Y1 cells

Mouse adrenal Y1 cells have been used extensively for the studies of regulation of steroidogenic gene expression, including the analysis of ferredoxin promoter. We therefore chose to analyze regulation of ferredoxin gene expression by various stimulators in Y1 using Northern-blot analysis. As shown in Fig. 1, cAMP was the major stimulator of ferredoxin synthesis. Angiotensin II and phorbol ester PMA slightly repress ferredoxin mRNA levels. Angiotensin II is known to alter calcium levels in the adrenal glomerulosa cells [10]. Phorbol esters modify protein kinase C activity which also alters intracellular calcium concentration [11]. Therefore, it seems that reagents which change intracellular calcium concentration may have a slight negative effect on ferredoxin levels.

The ferredoxin promoter has been located to contain a TATA and 2 Sp1 elements which function in cAMP-stimulated transcription [8], therefore whether the same element functions in regulation by other reagents was tested. The control region from -76 to -42 was cloned in front of an ovec vector driven by the β-globin minimal TATA promoter [9]. This promoter was greatly induced when 8-Br-cAMP was present (Fig. 2). Other reagents, such as synthetic glucocorticoid dexamethasone, A23187, and phorbol ester PMA, decreased basal transcription by about 20%. These reagents had the same effect whether alone or in combination, showing that they acted through the same pathway. There was an additive effect when A23187, phorbol ester, or dexamethasone was added together with 8-Br-cAMP, indicating 8-Br-cAMP used a different pathway for stimulation, and both pathways were independent of each other. The recapitulation of endogenous regulation by transfection experiment also demonstrated that the basal promoter contained all the control elements necessary for regulation.

Regulation of ferredoxin expression in H295 cells

A new cell line, NCI-H295, has recently been characterized [12, 13]. This cell line retains multiple steroidogenic function, including *P*450scc, *P*450c21, *P*450c17, *P*450c11, *P*450as, *P*450aro etc., in a regulated fashion. In addition, this human cell line will also be perfect for the study of human ferredoxin gene

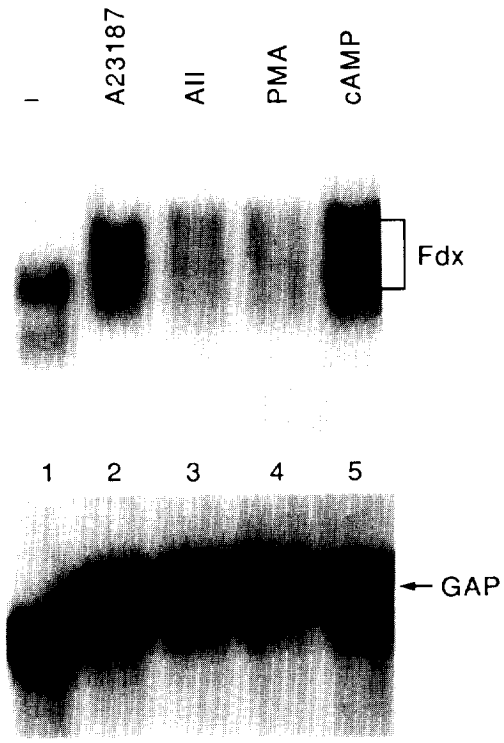

Fig. 1. Regulation of ferredoxin by calcium-inducing effectors in Y1 cells. Y1 cells were treated with 10 μM calcium ionophore A23187, 10 mM angiotensin II (AII), 100 μM phorbol ester PMA, or 1 mM 8-Br-cAMP for 2 h before RNA extraction. RNA was electrophoresed and hybridized with human ferredoxin cDNA probes (Fdx). The same blot was stripped and rehybridized with an internal control probe GAP.

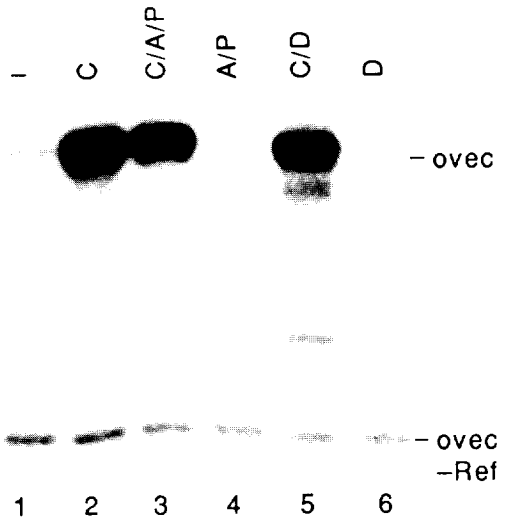

Fig. 2. Slight repression of A23187, phorbol ester, and dexamethasone on the Fdx-WT expression. Y1 cells were transfected with Fdx-WT and OVEC-Ref plasmids. A day later, the cells were split into 6, and were supplemented with nothing (–), 20 μM forskolin (C), 100 nM dexamethasone (D), 0.5 μM A239187 (A), 30 nM phorbol ester PMA (P), or their combinations. RNA was harvested 24 h later and was assayed for reporter gene expression by primer extension.

expression. Ferredoxin expression in H295 cells was inhibited by cycloheximide and stimulated by forskolin (Fig. 3). The combination of cycloheximide and forskolin also exhibited a negative effect, indicating that the stimulatory effect of forskolin was blocked by cycloheximide, the protein synthesis inhibitor. Expression of glyceraldehyde-3-phosphate dehydrogenase (Gap), which was used as an internal control, was not affected by the treatment. The effect of cycloheximide to block cAMP-stimulated ferredoxin gene expression was unique to H295 cells. In almost all other

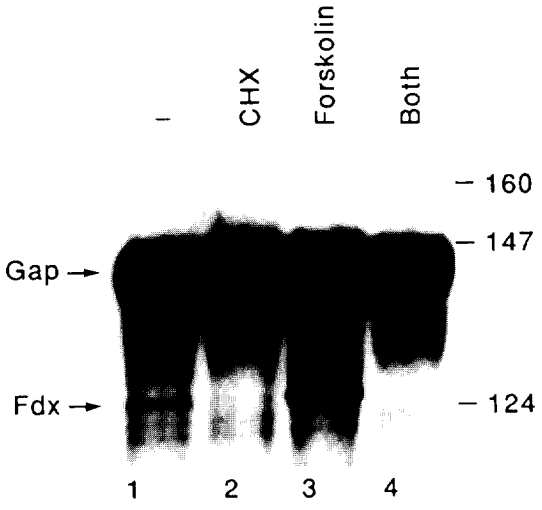

Fig. 3. Endogenous expression of ferredoxin gene in H295 cells. H295 cells were treated with nothing (–), cycloheximide (CHX), Forskolin, or both drugs for 24 h before RNA was harvested and analyzed by primer extension with both ferredoxin (Fdx) and Gap primers at the same time.

steroidogenic cell lines, such as granulosa cells [7], Y1 [6], or JEG-3 cells [2], the expression of ferredoxin was synergistically stimulated by cycloheximide and cAMP. Only in bovine adrenocortical cell culture [5], was the expression of ferredoxin inhibited by cycloheximide, as for all other steroidogenic *P*450s. Therefore, the fact that human H295 cells can suppress ferredoxin expression in the presence of cycloheximide shows that it retains a property more similar to the *in vivo* situation.

The promoter function of the human ferredoxin gene was tested in H295 cells. Using the same constructs as in Fig. 2, we observed an increase in test mRNA synthesis (ovec), in which either cycloheximide or forskolin was added to the cell culture. These two drugs also superinduced transcription when added together (Fig. 4). The empty ovec vector had a very low activity, which was detectable only when both forskolin and cycloheximide were added (Fig. 4, lane 4). Cycloheximide, but not forskolin, also has an effect on the internal control plasmid, ovec-Ref, which contains the SV40 promoter and enhancer. These data showed that the ferredoxin promoter, as determined by transfection into H295 cells, was superinduced by cycloheximide and differed from the endogenous expression, which showed cycloheximide sensitivity. Therefore, the transfection data did not parallel the endogenous expression. This reflects our limitation in using transfection experiments to study gene regulation.

Regulation of ferredoxin in nonsteroidogenic cells

The expression of ferredoxin was studied by Northern-blot analysis in different cell lines. Y1 and I-10 are two steroidogenic cell lines. Y1 has a low level of ferredoxin expression which was stimulated by 8-Br-cAMP (Fig. 5). I-10, the mouse testis Leydig-tumor cell line, had very little basal expression, but could be stimulated by the addition of 8-Br-cAMP (Fig. 5, Lanes 3 and 4). HeLa and COS-1, two nonsteroidogenic cell lines, expressed high levels of ferredoxin mRNA, yet they were not stimulated after 8-Br-cAMP treatment. It shows that the expression of ferredoxin in these nonsteroidogenic cells is constitutive, and not regulated by cAMP.

The function of the human ferredoxin promoter in nonsteroidogenic cell line was tested. Figure 6 shows the message levels transcribed from clones containing different lengths of the human ferredoxin 5′-flanking region fused to the chloramphenicol acetyltransferase (CAT) reporter gene and RNA transcribed from a constransfected CAT plasmid driven by Rous Sarcoma Virus (RSV) promoter served as an internal control. The CAT RNA transcribed from two promoters were marked respectively. In Y1 cells, promoter activity was readily detectable in the clone containing 94 bp of the 5′-sequence, and was stimulated by cAMP (Fig. 6). In Rat-1 fibroblast cells, the promoter function was even stronger than in Y1 when the clone was 94 bp long, yet

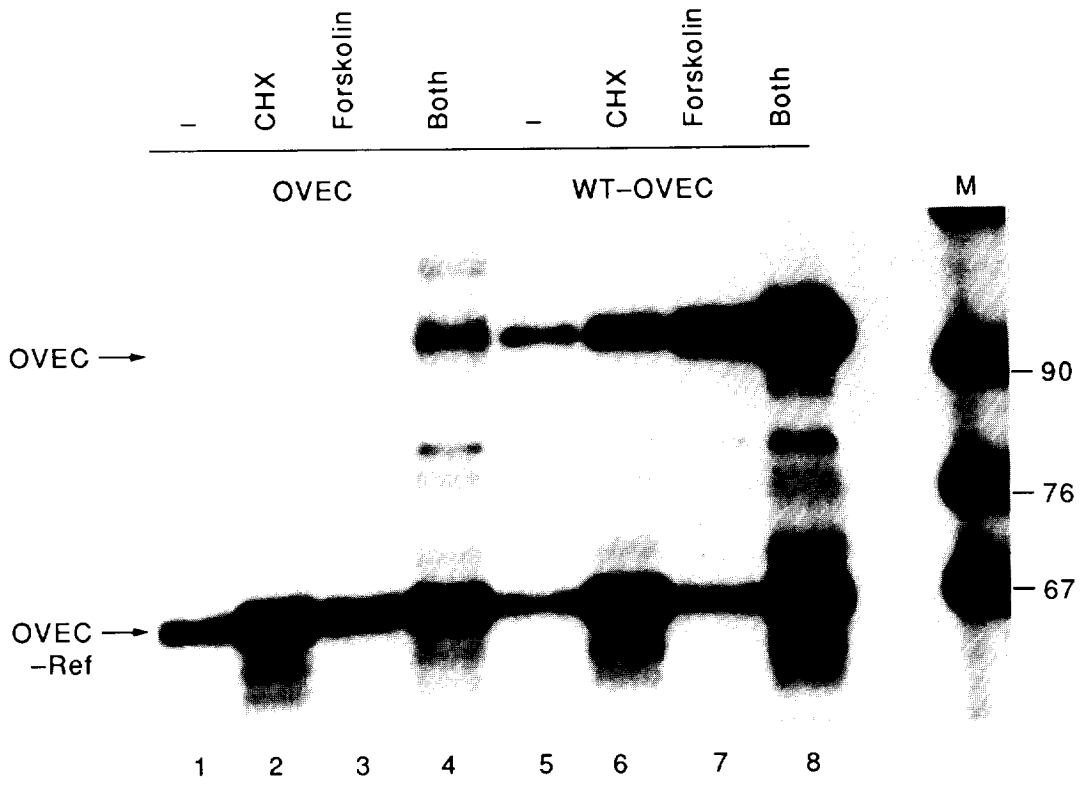

Fig. 4. Superinduction of Fdx-WT transcription in H295 cells by cAMP and cycloheximide. Fdx-WT plasmid in OVEC vector (WT-OVEC) was cotransfected with internal control plasmid OVEC-Ref into H295 cells. On the second day, cells were split into 4 plates, and were treated with nothing (−), 10 μg/ml cycloheximide (CHX), 40 μM forskolin, or both drugs for 24 h. RNA was analyzed by primer extension of a globin primer. Size marker was shown at the right of the gel.

it was not stimulated by cAMP, nor the longer clones. Therefore the ferredoxin promoter was active in both steroidogenic and nonsteroidogenic cell lines, but the cAMP stimulation was only steroidogenic cell specific.

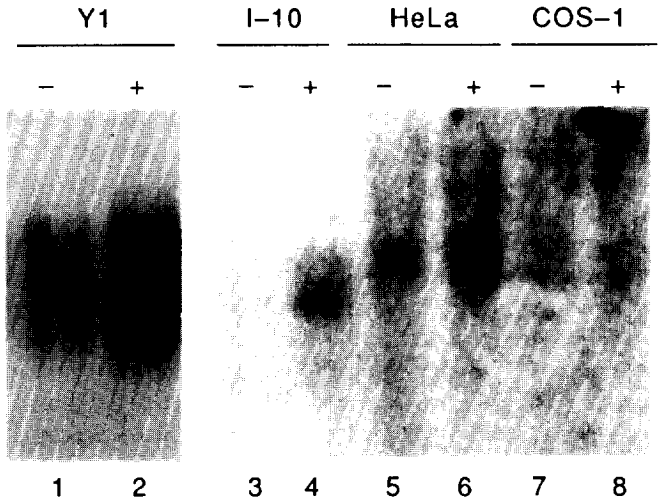

Fig. 5. Ferredoxin expression in nonsteroidogenic cells is not regulated by cAMP. RNA from Steroidogenic Y1, I-10, nonsteroidogenic HeLa, and COS-1 cells treated with (+) or untreated (−) with 1 mM 8-Br-cAMP was electrophoresed, blotted, and hybridized with radioactive human ferredoxin cDNA probe.

DISCUSSION

In this report, we have studied the regulation of the human ferredoxin gene in both steroidogenic and nonsteroidogenic cells. Although ferredoxin gene is expressed in both cell types, it is stimulated by cAMP only in steroidogenic cells. In addition, it was inhibited slightly by angiotensin II and phorbol ester PMA in Y1 cells. This active transcription in all cells, yet tissue-specific regulation by cAMP only in steroidogenic cells, is unique for ferredoxin. As ferredoxin is found in many tissues, yet only required to be regulated in steroidogenic tissues, it is reasonable that nature has devised a scheme to regulate ferredoxin only when necessary.

The human ferredoxin promoter started to function when it was about 94 bp long and contained one Sp1 site. When there are two Sp1 sites as in the 209 bp fragment, then there is full promoter function. This promoter is functional in both steroidogenic and nonsteroidogenic cells, consistent with the finding that Sp1 is present in all cell types. Yet, the promoter responds to cAMP to further activate transcription only in steroidogenic cells. Therefore the factors that participate in cAMP-dependent transcription are present only in steroidogenic cells.

The mode of ferredoxin expression was characterized in H295 cells. The stimulation of ferredoxin in H295

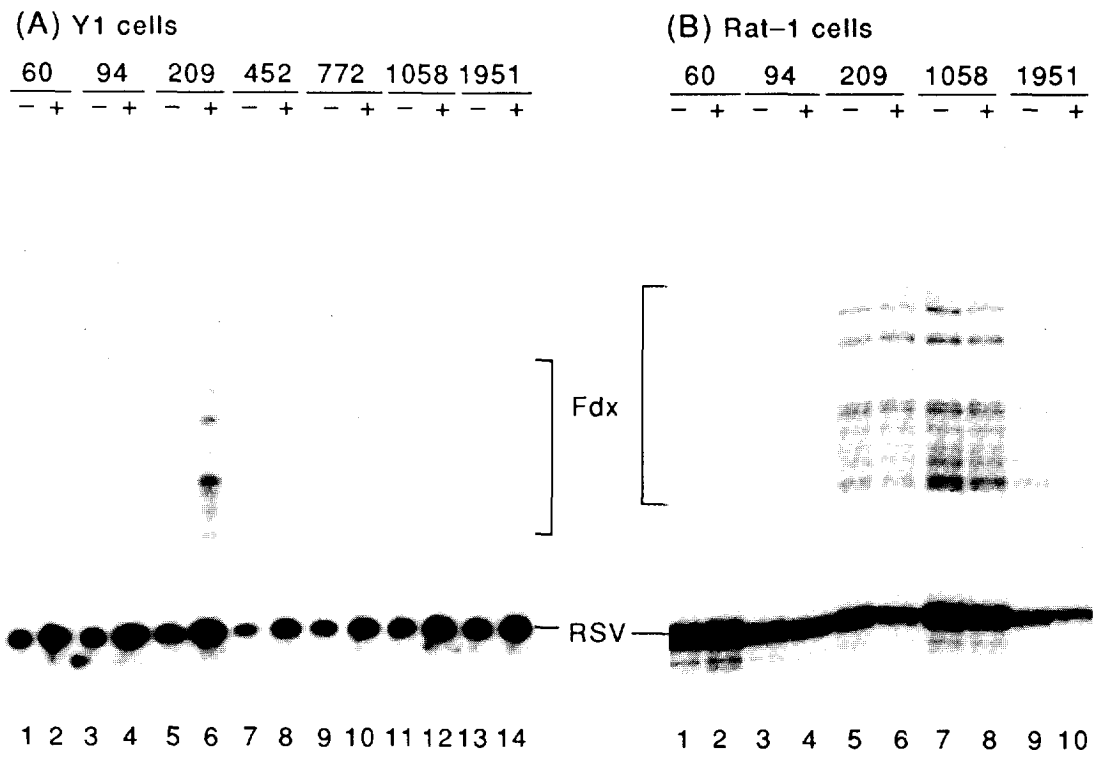

Fig. 6. The human ferredoxin promoter responds to cAMP in Y1 but not Rat-1 and COS-1 cells. Y1 (A) and Rat-1 (B) cells were cotransfected with internal control plasmid RSVCAT and plasmids carrying CAT gene driven by different lengths of the 5′-flanking region of the human ferredoxin gene as written on top of each lane. On the second day, each plate of cells was split into two, 20 μm forskolin was added to one plate (+) but not the other (−). The amount of CAT RNA was analyzed by primer extension using a CAT primer 24 h later. Transcripts driven by the RSV and ferredoxin (Fdx) promoters were marked respectively.

cells was suppressed by cycloheximide, a property of the cells similar to *in vivo* situation. Therefore, this cell line appears to be a good cell line to use because of its property and its human origin. We, however, found a discrepancy in results obtained from transfection experiments and endogenous expression. This is one example of problems encountered by transfection studies. It is possible that plasmid transfected transiently into cells was not incorporated into the chromosome yet, and was not in the same chromatin structure as most genes are *in vivo*. This could explain the lack of cycloheximide inhibition of transfected genes. Eventually approaches using transgenic mice will have to be taken to definitively answer the questions *in vivo*.

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