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Expression of biologically active hormone-sensitive lipase in mammalian (COS) cells

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cDNAs encoding rat adipose tissue hormone-sensitive lipase were expressed in COS cells, under the control of the SV40 promoter to half the level in rat adipocytes, the richest native source of the enzyme. A cDNA lacking most of the long 5'-untranslated region of the full-length rat hormone-sensitive lipase cDNA was, with regard to the lipase activity, on the average 70% more efficiently expressed than the full-length cDNA. The recombinant protein was almost identical to hormone-sensitive lipase of rat adipose tissue with respect to specific activity, susceptibility to inhibitors, molecular size, phosphorylation and activation by cyclic AMP-dependent protein kinase. The described eukaryotic expression system will allow analysis of effects of amino acid substitutions introduced into the lipase molecule by site-directed mutagenesis.

Triacylglycerol lipase; COS cell; Expression system

1. INTRODUCTION

Hormone-sensitive lipase (HSL) is a key enzyme in lipid metabolism and overall energy homeostasis in mammals. It catalyzes the rate-limiting step in the breakdown of stored triacylglycerols in adipose tissue, i.e. the hydrolysis of triacylglycerols to diacylglycerols [1,2]. HSL is also present in the steroidogenic tissues [3–6], where it functions as a cholesterol ester hydrolase and has been proposed to play an important regulatory role in the synthesis [3] and also hydrolysis [7] of steroid hormones from these tissues. The activity of HSL is under strict hormonal and neural control, a mechanism involving phosphorylation of a single serine residue (Ser-563) [6,8] by cylic AMP-dependent protein kinase [9,10]. In response to lipolytic hormones, such as noradrenaline, HSL is phosphorylated and concomitantly activated, whereas antilipolytic hormones, such as insulin, cause a net dephosphorylation and deactivation.

In addition to the phosphorylation site for activation by hormones, HSL has a second phosphorylation site, the basal phosphorylation site. Its phosphorylation by cyclic AMP-independent kinases does not directly affect HSL activity [10]. The basal phosphorylation site has recently been identified as Ser-565 in the rat sequence [11]. Its extact function has not been established, but recently it has been proposed to have a regulatory, antilipolytic function since its phosphorylation, catalyzed by AMP-activated kinase, prevents the subsequent phosphorylation of Ser-563 by cylic AMP-dependent protein kinase [11,12]. The HSL catalytic site has not been elucidated. However, HSL is inhibited by diisopropylfluorophosphate (DFP) (1) and related compounds, suggesting that a reactive serine is involved. One possible candidate for this reactive serine is Ser-423, which is found in the sequence GDSAG. The sequence motif GXSXG is found at the active centers of most serine proteases [13] and has been reported in the sequence of all known mammalian and bacterial lipases [14].

To identify the catalytic site of HSL, and to establish the functional role of the basal phosphorylation site, experiments employing the site-directed mutagenesis technique are urgently required. In this communication, we report on the development of a eukaryotic expression system for HSL which allows efficient expression of apparently native, catalytically active enzyme, the activity of which can be controlled through phosphorylation by cylic AMP-dependent protein kinase. This expression system should be valuable for site-directed mutagenesis experiments to finally identify the catalytic site. It should also be useful for determining the role of the basal site phosphorylation in the hormonal regulation of HSL activity.

Abbreviations: HSL, hormone-sensitive lipase; FLHSL, full-length HSL cDNA; F5⁻HSL, HSL cDNA lacking the 5'-untranslated region; LPL, lipoprotein lipase; CAT, chloramphenicol acetyl transferase; IgG, immunoglobulin G; DFP, diisopropylfluorophosphate; anti-HSL, the IgG fraction of a rabbit polyclonal antiserum prepared using rat adipose tissue HSL lipase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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2. EXPERIMENTAL

2.1. *Materials*

Restriction enzymes and DNA modifying enzymes were purchased from BRL, except for *Bsm*I, which was obtained from Stratagene. The eukaryotic expression vector pSVL was obtained from Pharmacia and the shuttle vector Bluescript from Stratagene. Plasmid DNAs were isolated using the 500 packs from Qiagen.

2.2. *DNA constructs*

A full-length rat HSL cDNA (FLHSL) was constructed from three overlapping cDNA fragments [6,15] and ligated into the pSVL vector at the *Sma*I site (Fig. 1). A second construct (F5$^-$HSL) lacking all but the last 13 (of 614) 5'-untranslated nucleotides was made utilizing a unique Bsm I site in FLHSL. After end filling with T4 DNA polymerase [16], this construct was ligated into the *Sma*I site of pSVL (Fig. 1). Similarly, pSVL-LPL contains the coding sequences for human LPL [17] cloned directionally into the *Xho*I and *Bam*HI sites of pSVL.

pKTSV [18] is a pUC 18 based plasmid construct which carries an SV40 origin of replication and expresses CAT activity under the regulation of the SV40 early promotor.

2.3. *Transfection*

COS cells, approximately 80% confluent, were transfected with the different DNA constructs using 30 μg Lipofectin (BRL) per 60 mm tissue culture plate following instructions provided by the manufacturer. 72 h after transfection, cells to be analyzed for RNA were harvested by adding the denaturing solution [19] directly to the plates. Cells for other analyses were rinsed with 3 ml of 0.9% NaCl and then scraped off in 1.5 ml of 0.9% NaCl using a rubber policeman. The cells were pelleted by a brief centrifugation at room temperature in a microfuge and then homogenized in 3–5 vols. of 0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 1 mM benzamidine, 20 μg/ml antipain and 20 μg/ml leupeptin using a motordriven homogenizer with a teflon pestle. In some experiments, the COS cell homogenates were spun at 110000×*g*, 45 min, 4°C, and the supernatant used for further analysis. A fat-depleted 110000×*g* infranatant of a rat epididymal adipose tissue homogenate was prepared [1] from 200 g male Sprague-Dawley rats (ALAB, Sweden), fed ad libitum.

2.4. *Enzyme assays and activation by cAMP-dependent protein kinase*

HSL was routinely assayed using 1(3)-mono[^{3}H]oleoyl-2-oleylglycerol (a diacylglycerol ether analogue) as substrate [20]. One unit (U) of enzyme activity is defined as 1 μmol of fatty acid released per min at 37°C. Enzyme assays in the presence of HSL inhibitors were performed as described [5]. HSL in homogenates (see 2.3) was activated by cylic AMP-dependent protein kinase by incubation at 37°C for 30 min with 0.5 mM ATP, 5 mM $MgCl_2$, 1 mM dithioerythritol, 10 mM Na-PP_i and the catalytic subunit of cyclic AMP-dependent protein kinase (3 μg/ml), purified to near homogeneity from rat adipose tissue [9,21]. In controls, the protein kinase was omitted. Activated and control samples were assayed using a 0.5 mM trioleoylglycerol substrate [22] at a pH of 8.3 [9].

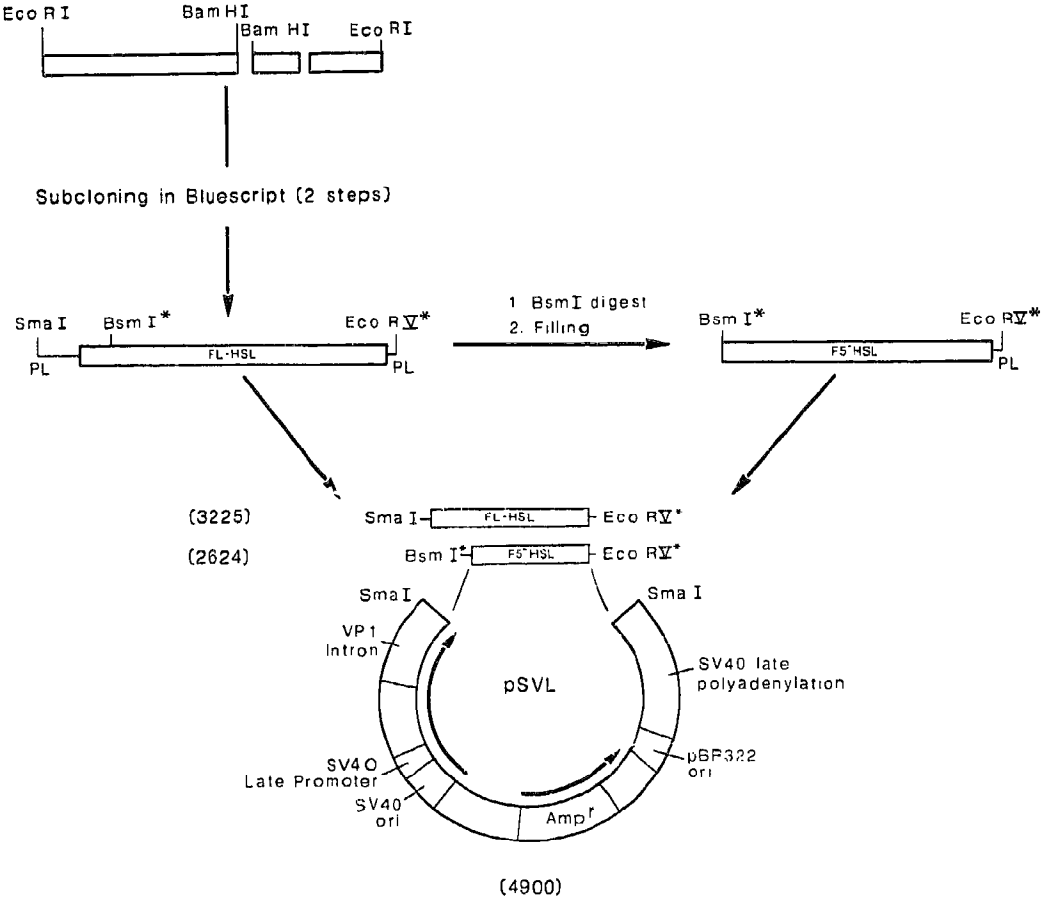

Fig. 1. Construction of plasmids. Three cDNA fragments (boxed) were subcloned into Bluescript to obtain a fullength cDNA construct (FL-HSL). F5$^-$HSL was then obtained as illustrated and the two different constructs were subcloned into the *Sma*I-site of pSVL. PL indicate the polylinker of Bluescript. Restriction sites labeled with an asterisk were not reconstituted during the procedure. Figures in parentheses indicate number of nucleotides in constructs.

2.5. *Phosphorylation and immunoprecipitation*

Aliquots of 110000×*g* supernatants or infranatants (see 2.3) were phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase as described above for the activation, except that 0.1 mM [γ-^{32}P]ATP was used and the Na-PP$_i$ was exchanged for 100 mM NaF. HSL was then immunoprecipitated twice with anti-HSL [23,24]. Phosphorylated aliquots, corresponding to approximately 50 ng HSL protein, were immunoprecipitated with 150 μg anti-HSL (overnight 4°C) and 45 mg of extensively prewashed *S. aureus* protein A (Sigma) (30 min, 4°C). After centrifugation (10000×*g*, 5 min, 4°C), the immunoprecipitates were washed 3 times with 1 ml of phosphate-buffered saline, 0.1% (w/v) *N*-lauroyl sarcosine, and dissolved in 60 μl of 0.1 M Tris-HCl, pH 7.5, 2% (w/v) SDS by boiling. After adding 165 μl of 0.3 M Tris-HCl, pH 7.5, 45 μl of 20% (w/v) Triton X-100 and 30 μl of 10% (w/v) *N*-lauroyl sarcosine, a second immunoprecipitation was performed as above. Throughout the immunoprecipitations and washings NaF was added to 100 mM to inhibit protein phosphatases. The final immunoprecipitates were dissolved by boiling in 50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 2% (w/v) β-mercaptoethanol and 5% (w/v) glycerol.

2.6. *Extraction of RNA and Northern blot analyses*

Total RNA was isolated using a single-step acid guanidinium thiocyanate/phenol/chloroform extraction [19]. RNA samples were electrophoresed in 1% agarose, 2.2 M formaldehyde gels, transferred to nylon membrane (Schleicher & Schuell) and cross-linked to the membrane by exposure to ultraviolet light [25]. The blots were hybridized with a 1.9 kb rat HSL cDNA probe [6,14], radiolabeled with [^{32}P]dCTP to a specific activity of about 10^9 cpm/μg [26], in 0.5 M sodium phosphate (pH 7.0), 1% (w/v) bovine serum albumin, 1 mM EDTA and 7% (w/v) SDS at 60°C overnight. The blots were washed twice (20 min each wash) in 15 mM NaCl, 1.5 mM trisodium citrate, 0.1% SDS at 60°C and exposed to Kodak XAR 5 film.

2.7. *Other analyses*

CAT assay was performed essentially as described in [27] using aliquots of the same homogenates as used for HSL activity measurements (see 2.3). The autoradiograms of the thin layer chromatography plates were quantitated by densitometric scanning (Joyce-Loebl, Chromoscan 3) at 626 nm. The activity was calculated as follows: percent acetylated = (peak areas of acetylated species)/(peak area in acetylated species + counts in nonacetylated chloramphenicol). The CAT activity of the pSVL transfected cells was defined as 1 and the CAT activity of other cells normalized against this.

Protein was determined according to Bradford [28] using bovine serum albumin as standard. SDS-PAGE was performed in slab gels (14×14 cm) according to Laemmli [29] with modifications [30]. Reference proteins were myosin (M_r, 205000), β-galactosidase (116000), glycogen phosphorylase a (97000), human transferrin (76000), bovine serum albumin (66000), ovalbumin (45000) and carbonic anhydrase (29000) (all from Sigma). Western blotting was performed as described in detail in [23] using anti-HSL. The Western blots were quantitated by densitometric scanning of the autoradiograms.

3. RESULTS

3.1. *Expression of HSL in COS cells*

A full-length rat HSL cDNA (FLHSL) was ligated into the pSVL vector (Fig. 1), a eukaryotic expression vector containing the SV40 late promoter and the SV40 late polyadenylation signal [31,32] (Fig. 1). Homogenates of COS cells, transfected with the pSVL-FLHSL construct, exhibited a more than 10-fold increase in diacylglycerol lipase activity, compared to COS cells transfected with the pSVL vector alone or the pSVL vector containing a cDNA for human LPL [17] (Fig. 2, filled bars).

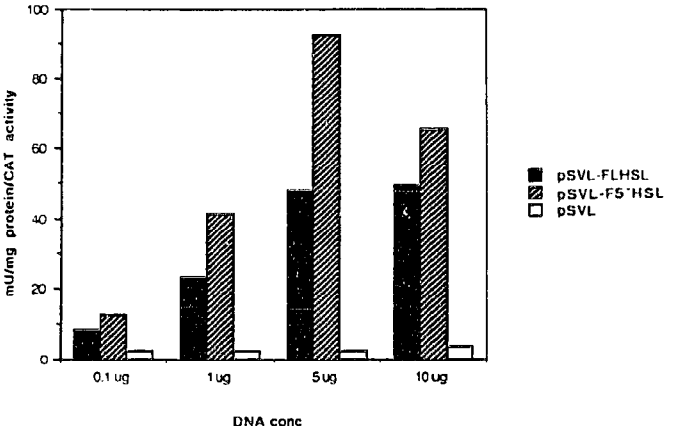

Fig. 2. Optimization of HSL expression in transfected COS cells. Duplicate plates were transfected with pSVL alone, pSVL-FLHSL or pSVL-F5$^-$HSL at 0.1 μg, 1 μg, 5 μg or 10 μg per plate and in all cases cotransfected with 0.1 μg pKTSV. At day 3 the cells were harvested and homogenates prepared and analyzed for HSL activity, total protein and CAT activity (for details, see section 2). Shown is the mean HSL activity per mg total protein per CAT activity.

To examine if the expression of HSL in the COS cells could be enhanced by removing most of the long 5′-untranslated region, a construct was made lacking all but 13 nucleotides of the 5′-untranslated region (F5$^-$-HSL) (Fig. 1). At all DNA concentrations examined, the cDNA lacking 5′-untranslated sequences expressed higher levels of HSL than the full-length cDNA (Fig. 2).

The reproducibility of the transfection procedure was analyzed by performing transfections in quintuplicates with 1 μg each of the pSVL-FLHSL, pSVL-F5$^-$-HSL and pSVL constructs. The HSL activity of the cell homogenates, expressed as mU/mg total protein/CAT activity were found to be 11.8±2.4 for pSVL-FLHSL, 30.5±2.6 for pSVL-F5$^-$HSL and 4.2±0.4 for pSVL (mean±SD).

The HSL mRNA species found in cells, transfected with the different HSL cDNA constructs, were analyzed by Northern blot analysis. Cells transfected with the pSVL-FLHSL and pSVL-F5$^-$HSL constructs expressed major HSL mRNA species with apparent sizes of 3.8 kb and 3.7 kb, respectively (Fig. 3). The amount of HSL mRNA, by densitometric scanning of the autoradiogram, was approximately 60% higher for the pSVL-F5$^-$HSL construct than for the pSVL-FLHSL construct, consistent with the approximately 80% higher expression of HSL protein (Fig. 2, 1 μg value). The HSL transcripts expressed in COS cells have an apparent size about 500 nucleotides larger than rat adipose tissue HSL mRNA, presumably due to untranslated sequences added by the pSVL construct.

3.2. *Characterization of the expressed HSL protein*

The HSL activity of the transfected COS cells was analyzed for susceptibility to known HSL inhibitors [1]

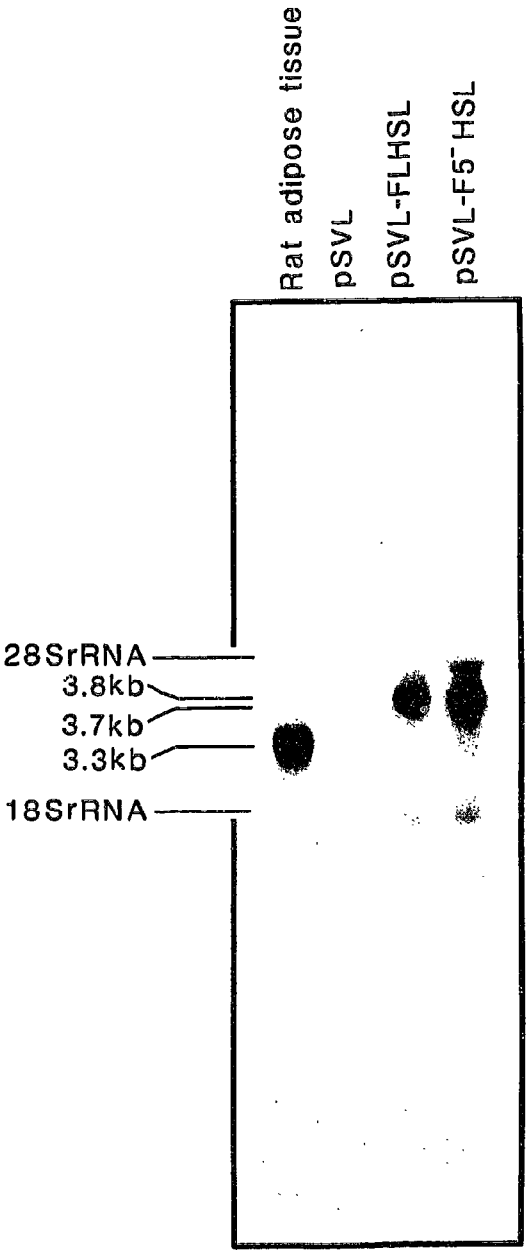

Fig. 3. Northern blot analysis of COS cells transfected with different pSVL-HSL constructs. Polyadenylated RNA (1 μg) from rat adipose tissue or 10 μg of total RNA from COS cells transfected with 1 μg of either pSVL, pSVL-FLHSL or pSVL-F5⁻HSL were electrophoresed in a 2.2 M formaldehyde, 1% agarose gel and blotted to nylon membrane. The blot was hybridized with a ^{32}P-labeled rat HSL cDNA probe, washed and exposed to film for 16 h (for details, see section 2). The indicated sizes are calculated from mobility of a RNA size standard (BRL).

including DFP, Hg^{2+} and anti-HSL [23]. It was found that the HSL activity of COS cells, transfected with either pSVL-FLHSL or pSVL-F5⁻HSL, was inhibited by these inhibitors to approximately the same extent as rat adipose tissue HSL (Table I). The low diacylglycerol lipase activity of cells transfected with the pSVL alone, on the other hand, was inhibited much less (Table I) indicating that it was due to another lipase.

To address the question of how much of the expressed protein was catalytically active, the amount of HSL polypeptide in the cells was estimated from Western blots, using the 110000×*g* infranatant of a rat epididymal adipose tissue homogenate (see above) as reference. The amount of catalytically active HSL was estimated, by densitometric scanning, to be between 75% and 80% (range for 2 different transfection experiments), with no difference between FLHSL and F5⁻HSL (data not illustrated).

The ability to phosphorylate expressed HSL using cyclic AMP-dependent protein kinase was examined in 110000×*g* supernatants of homogenates of cells, transfected with either pSVL-FLHSL or pSVL-F5⁻HSL. Immunoprecipitates of ^{32}P-labeled HSL showed that the expressed HSL protein could be phosphorylated to approximately the same extent as rat adipose tissue HSL, when using comparable ratios between the lipase and the kinase (Fig. 4). The exact degree of phosphorylation was not determined but it could be estimated, based on scanning densitometric analysis of the autoradiogram in Fig. 4 and taking into account the presence of some catalytically inactive protein in the pSVL-FLHSL homogenate (20% for the experiment in Fig. 4), to be almost 90% of that found for rat adipose tissue HSL. Phosphorylation in the presence of unlabeled ATP and assaying trioleoylglycerol lipase activity demonstrated that the HSL phosphorylation by cyclic AMP-dependent protein kinase increased enzyme activity by 46% (46±13%, mean±SD for $n=5$ with the same COS cell homogenate). This was comparable to the activation of a fat-depleted rat adipose tissue homogenate, which was 56% (56±9%, mean±SD, $n=5$) under the same conditions, and is in agreement with previous findings for activation of crude homogenates [33].

Table I

Inhibition of diacylglycerol lipase activity in transfected COS cells by HSL activity inhibitors

Additions	Diacylglycerol lipase activity (% remaining)			
	pSVLFL-HSL	p-SVL-F5⁻HSL	pSVL	Rat adipose tissue
anti-HSL	25.1±4.6	18.8±4.2	64.5± 4.3	11.0±0.5
DFP (100 μM)	2.5±0.1	1.2±0.3	48.6±20.4	6.4±1.6
Hg^{2+} (100 μM)	3.9±1.0	2.1±0.9	19.1±11.3	6.2±0.6

Homogenates of COS cells transfected with 5 μg of different pSVL constructs as indicated and a homogenate of rat adipose tissue were assayed for diacylglycerol lipase activity after preincubation with anti-HSL (whole antiserum, diluted 10-fold), DFP or Hg^{2+}, at the indicated concentrations. Lipase activity is expressed as percent of the activity after preincubation with the appropriate control in each case. Values are mean±S.D. (n=3).

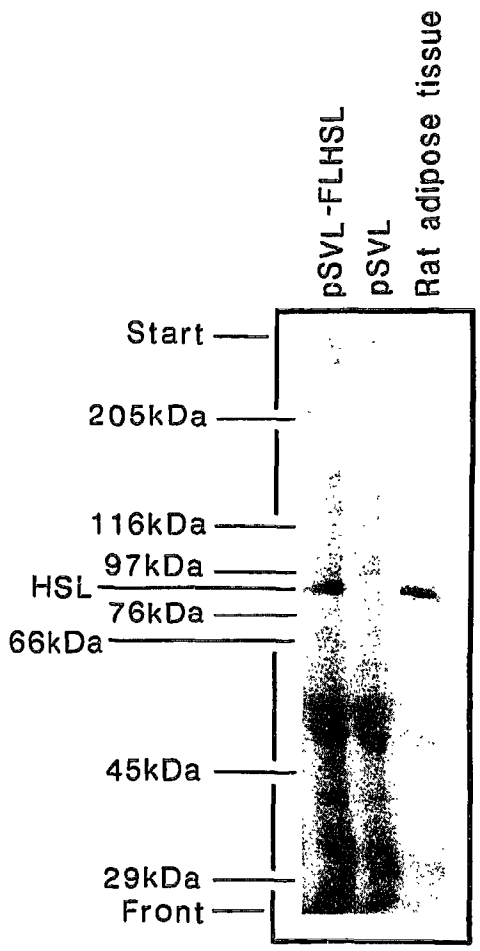

Fig. 4. Phosphorylation of COS cell-expressed HSL by cAMP-dependent protein kinase. Aliquots of 110000×g supernatants of COS cell homogenates, transfected with either pSVL-FLHSL or pSVL alone, and an aliquot of an 110000×g infranatant of rat adipose tissue were phosphorylated with [^{32}P]ATP-Mg^{2+} and cAMP-dependent protein kinase (the catalytic subunit). After two immunoprecipitations with anti-HSL and *S. aureus* protein A, the immunoprecipitates were subjected to SDS-PAGE (8%) followed by autoradiography for 6 days. The positions of the reference proteins are indicated on the lefthand side of the diagram.

4. DISCUSSION

The expressed HSL was almost identical to HSL from rat adipose tissue with respect to all examined enzymological and physicochemical properties. The expressed HSL activity (activity per mg total protein) was, with the optimal cDNA construct and DNA concentration (F5$^-$HSL, 5 μg), nearly half of that seen in adipose tissue from 200 g rats. The transfection efficiency has not been determined, but based on the original report describing the use of Lipofectin for transient transfections [34], it is reasonable to assume that it is close to 100%, and that the specific activities measured therefore are close to the specific activity of the HSL-expressing cells. Removal of 601 of the 614 5′-untranslated nucleotides resulted in a more efficient expression of HSL compared to the FLHSL (Fig. 2). The increase in expression was between 45% and 90% depending on the DNA concentration used. The fact that the increase appeared to be similar at the mRNA and protein level suggests an increased transcription rate or an increased mRNA stability, rather than an effect on translation. The sizes of the HSL mRNA species in the transfected COS cells, 3.8 and 3.7 kb for FLHSL and F5$^-$HSL, respectively, are approximately what could be expected, considering the size of the HSL cDNA introduced in each case plus the additional 3′-untranslated sequences from the pSVL vector and a poly(A) tail. One slightly larger and one smaller hybridizing mRNA were also found. These mRNAs probably represent unspliced and degraded HSL mRNA, respectively, since they were absent from cells transfected with only pSVL.

The expressed HSL exhibited the same inhibition characteristics as rat adipose tissue HSL, and was phosphorylated by cylic AMP-dependent protein kinase concomitant with an increased enzyme activity. It was estimated that at least 50% of the expressed protein was catalytically active, and thus most likely in its true native conformation. The fact that the degree of phosphorylation was higher than the degree of catalytically active protein, can probably be explained by phosphorylation also of catalytically inactive protein, since it has been observed that denatured HSL, which has been immobilized on a nitrocellulose membrane after SDS-PAGE, can be phosphorylated to approx. the same extent as native HSL (C. Holm, unpublished observation). The low diacylglycerol lipase activity seen in COS cells transfected with the pSVL alone, and also in non-transfected COS cells (not illustrated), is at least to a large extent, due to a neutral esterase/lipase other than HSL. Indeed, these cells did not express an HSL mRNA and the diacylglycerol lipase activity found was inhibited to a considerably lower extent with known HSL inhibitors, especially anti-HSL.

The mammalian cell system described in this report for expression of apparently native HSL will allow site-directed mutagenesis experiments to be performed in order to identify the catalytic site and to determine if the basal phosphorylation site, as recently proposed [11], has a role in the hormonal regulation of HSL in adipocytes.

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