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Heterologous systems for expression of mammalian sulfotransferases

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Abstract

This paper describes the use of both mammalian and bacterial expression systems as tools to study the structural and functional relationships of proteins encoded by cDNAs to both rat and human aryl sulfotransferases. In particular, we describe the use of the mammalian COS cell system for functional expression studies, and the use of *Escherichia coli* for the expression and purification of a sulfotransferase fusion protein suitable as an antigen for the generation of sulfotransferase antibodies.

Keywords: Sulfotransferases; Heterologous systems; COS cells; *Escherichia coli*

Sulfotransferases (STs) comprise a superfamily of structurally and functionally related isoforms which are involved in the sulfation of a diverse range of exogenous and endogenous compounds including biogenic amines, steroids, hormones, and a variety of xenobiotic compounds including drugs and carcinogens [1]. Although generally thought of as a detoxification pathway, sulfation can also be involved in the bioactivation of certain drugs to their therapeutically active form, such as minoxidil, and some N-hydroxy aromatic amines to their ultimate carcinogenic species [1,2]. In order to cope with substrates of such diverse chemical structure, multiple forms of STs have evolved. To date at least six different aryl STs and seven different forms of steroid/bile acid STs have been purified or characterised from rat liver [1]. While purification studies clearly demonstrate that multiple forms of ST exist, it has not been possible to totally charactersise this enzyme system using this approach. This is due to the fact that STs may often show identical mobilities on SDSpolyacrylamide gels and near identical protein sequences thus leaving doubt as to the purity of these preparations. In addition, limitations often associated with obtaining sufficient material from tissues or concern with the tedious process of conventional low-yielding purification procedures may ultimately affect enzyme quantity and quality. The limited availability of human tissue specimens is also likely to hamper the study of human STs, particularly the extrahepatic forms.

An approach utilising molecular biology techniques can overcome many of these difficulties and has been used effectively to unravel the cytochrome P450, UDP-glucuronosyltransferase and glutathione transferase enzyme systems. For example, STs can be cloned, their cDNAs then inserted into eukaryotic or prokaryotic expression vectors and used to express active enzyme in cell culture, in techniques commonly known as cDNA-expression or heterologous expression. Enzyme activities and substrate specificities can then be analysed for the individually expressed ST isoforms with the knowledge that enzyme purity and integrity have not been compromised. With the recent availability of cDNAs encoding mammalian STs, heterologous expression now provides a means whereby these individual STs can be functionally and structurally characterised in detail.

In this report we describe the use of the COS cell system to study the function of a rat [3] and human [4] aryl ST sharing 79% amino acid similarity. In addition we describe the use of an *Escherichia coli (E. coli)* expression system suitable for the purification of large quantities of a rat aryl ST as an antigen for the production of antibodies.

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COS cell expression system

A number of heterologous systems are currently available to express proteins from their respective cDNAs including yeast, insect cells and *E. coli.* However, the mammalian COS cell expression system has been the most extensively used for the study of ST enzymes [3,5-7]. A major advantage of the COS cell expression system is the ease of expressing functional protein and the low background of endogenous ST activity of the COS cells. Other advantages of this system are that it is a mammalian cell vector system and the process is simple and rapid. The COS cell line was derived by integration of an origin-defective mutant of simian virus 40 (SV40) viral DNA into the genome of African green monkey kidney cells [CV-1] [8]. The resultant COS cells produce SV40 T antigen which is required for viral replication. Thus, any transiently transfected plasmid DNA which contains the SV40 origin of replication is capable of replicating in COS cells. Transfected cells, therefore, will contain multiple copies of the plasmid which can synthesise the protein encoded by the inserted cDNA. However, a disadvantage of the COS system is that protein expression is generally maximal 48-72 h post-transfection and presumably due to the excessive burden placed on the transfected cells by the replicating plasmid, the cells eventually die (i.e the system is transient).
A number of expression vectors have been used to successfully express cloned cDNAs in COS cells including the SV40 based vectors, pSVL (Pharmacia), pSVSPORT 1 (Gibco BRL) and p91023(B) [9], and the pCMV based vectors such as pCMV5 [10], as used in our laboratories. The pCMV mammalian expression vectors contain the promoter regulatory region of the human cytomegalovirus (Towne strain) major immediate early gene and are designed for use in essentially all cultured cells. They work extremely well in SV40-transformed simian COS cell lines and compared to the SV40-based vectors have been shown to give approximately a 5-10-fold greater level of expressed proteins including cytochrome P450s [11]. For this reason we chose to use the pCMV5 vector for expressing our cloned ST cDNAs. This vector was kindly provided by Dr Mark Stinsky, Department of Microbiology, School of Medicine, University of Iowa.

A rat liver aryl ST cDNA was cloned from a rat liver Lambda cDNA library using the polymerase chain reaction technique as recently described by us [3]. To functionally characterise this ST, it was subcloned into the pCMV5 COS cell expression vector and expressed in COS-7 cells [3]. The cDNA-expressed ST protein catalysed the sulfation of phenol and p-nitrophenol as well as the monoamine neurotransmitter dopamine, and the common food additive, vanillin. The kinetics of sulfation of these substrates by this COS-expressed ST were best described by single enzyme Michaelis-Menten kinetics. The apparent Km for phenol, p-nitrophenol, vanillin and dopamine were 0.87 #M, 0.27/~M, 7.75/~M and 44.3/zM, respectively. For the substrates, phenol and dopamine, these apparent Kms were quite similar to the apparent Kms obtained in rat liver cytosol (phenol 1.54/zM; dopamine 19.1 tzM) [3]. Activities in the COS cells transfected with vector alone (minus ST cDNA insert) were barely above background activity indicating the suitability of this expression
system for determining enzyme function.

We have also used the COS cell expression system to characterise a human liver aryl ST cDNA [4]. The protein encoded by this cDNA shares 79% similarity to the rat liver aryl ST characterised above. The human COS-expressed ST, similarly to the expressed rat enzyme, could also catalyse the sulfation of the substrates phenol, pnitrophenol and dopamine. Kinetic analyses of these reactions showed the apparent Kms for these substrates (phenol 3.4/~M, p-nitrophenol 0.6/zM and dopamine 345 /~M) by the recombinant human ST were generally higher than for the recombinant rat ST, being almost 8-fold higher in the case for dopamine. These data indicate that even though there appear to be functional similarities between these expressed rat and human aryl STs subtle differences are apparent and hence caution should be exercised in extrapolating results with ST proteins from one species to another. The catalytic characteristics of the human enzyme, with a high affinity (low Kin) towards simple phenols and low affinity (high Km) towards monoamines such as dopamine, indicates that the recombinant protein is functionally quite similar to the phenolmetabolising form of phenol ST (P-PST) partially purified from human liver by other workers [12]. The other form of phenol ST characterised in human tissues, the monoamine-metabolising form (M-PST) [9,10], has quite high affinity for sulfating monoamines. Other means to differentiate these two human PST forms include the use of the ST inhibitor 2,6-dichloro-4-nitrophenol (DCNP) as well as thermal stability studies [12,13]. P-PST is sensitive to inhibition by DCNP and is relatively thermostable. In contrast, M-PST is quite resistant to DCNP inhibition and is relatively thermolabile. Preliminary experiments performed with the COS-expressed human ST described above indicate that it is sensitive to inhibition by DCNP as well as being relatively thermostable. These studies together with the substrate kinetic data described above are consistent with this human ST cDNA encoding P-PST.

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E. coli expression system

A particular limitation of the COS cell system is the low level of enzyme expressed, requiring the availability of sensitive assays to measure enzyme function. Furthermore, it is relatively expensive and tedious to obtain large amounts of enzyme using mammalian cell culture. More recently, bacterial expression systems have been developed for the expression of many proteins (including the xenobiotic metabolis- ing enzymes), with these generally being devoid of many of the limitations associated with mammalian expression systems [14]. Expression levels obtained in *E. coli* have been reported as high as 50-100 mg per 1 of culture for some proteins, this being several orders of magnitude higher than would be obtained in COS cells.

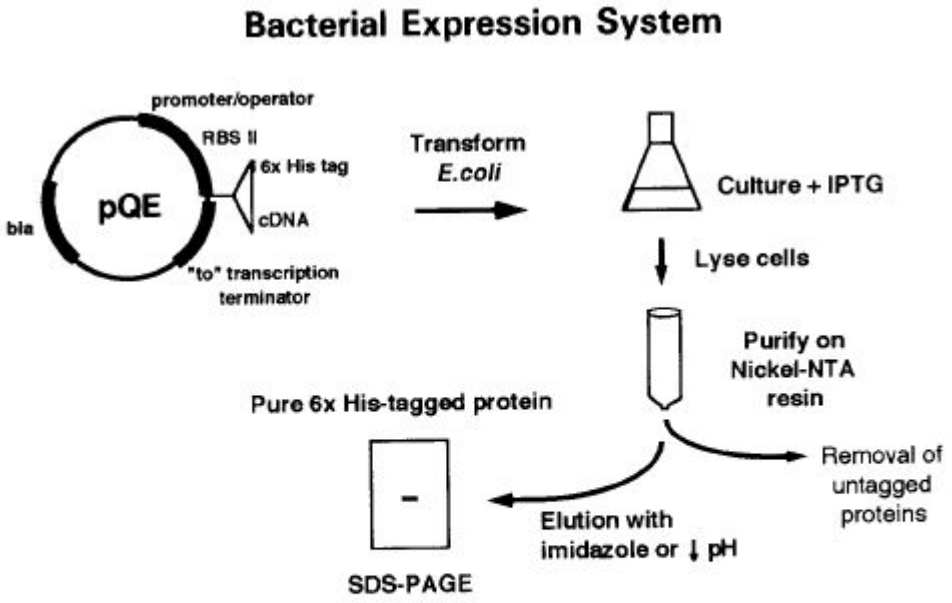

Fig. 1. Strategy for the expression and purification of 6 × His-tagged recombinant proteins in *E. coli*. Coding sequence (cDNA) of interest is inserted into the pQE expression vector (Qiagen) and the construct transformed into *E. coli*. Expression of tagged protein is induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG), the cells lysed and the cleared lysate containing tagged protein mixed with the Nickel-NTA resin. The resin is washed in a column (or in batch format) to remove contaminants, and the pure protein is eluted under gentle conditions by either reducing the buffer pH slightly (from pH 6 → pH 4) or adding imidazole as a competitor.

We were primarily interested in using *E. coli* as an expression system to synthesise sufficient ST protein such that it could be purified from other *E. coli* proteins and used as antigen for preparing antibodies. The availability of an antibody would enable us to measure the expression of ST proteins in a number of systems including, tissue preparations incorporating Western blotting and immunohistochemistry techniques, as well as detecting expression of ST protein in eDNA transfection experiments such as in the COS cells described above. This approach of generating antibodies again avoids all the problems associated with having to purify protein from animal or human sources as described earlier. We used the *E. coli* expression system 'QIAexpress', available commercially in 'kit' form (Qiagen Inc., Chatsworth, CA, USA), for the expression and purification of recombinant proteins (Fig. 1). An ideal feature of this system is the ability to purify the expressed protein from other *E. coli* proteins in a one step procedure. This is achieved by the addition of an affinity tag of six consecutive histidine residues (6 x His tag) to the expressed protein which can then be purified on the unique Nicket-NTA (Nitrilo-Tri-Acetic acid) resin which has remarkable selectivity for proteins with a 6 x His tag. This means that tagged proteins can be efficiently purified to greater than 95% purity in just one step. To investigate the suitability of this system for ST proteins we used the rat aryl ST eDNA previously isolated by us [3]. This eDNA was inserted into the 'QIAexpress' bacterial vector pQE-11 (this vector has the 6 x His tag sequence incorporated upstream of the multiple cloning site) and transformed into the *E. coti* strains M 15[pREP4] and SG13009[pREP4] provided (Qiagen). Protein expression from the pQE vector was induced by the addition of isopropyl-~3-D-thiogalactopyranoside (IPTG) and purification was generally as per the manufacturers instructions (see Fig. 2 legend for details). However, denaturing conditions had to be used to solubilise the expressed ST protein, which appears to be present within insoluble inclusion bodies in the *E. coli.* This required the use of 6M guanidine HC1, but because the interaction between the Ni-NTA resin and the 6 x His tag is independent of protein structure, purification is unaffected by strong denaturants. This is a clear advantage of the above *E. coli* expression and purification system over others such as pGEX (Pharmacia) which use glutathione S-transferase as the affinity 'tail' and cannot be used under such denaturing conditions.

The results of a typical ST expression and purification protocol are shown in Fig. 2. As can be seen from this figure the level of expression we obtained was high (in the order of 25 mg purified rat ST protein per 500 ml culture were obtained), as evidenced by the marked induction of ST protein (~ 41 kDa) by IPTG, and from the SDS-PAGE analysis the eluted protein appears greater than 95% pure. The purified recombinant ST protein was used as antigen to generate antibodies in rabbits, and as can be seen in the Western immunoblot in Fig. 3, is suitable for detecting the expression of ST protein in rat cytosolic preparations and in COS cell expression experiments. It should be noted that the 6 x His tagged ST protein detected in Fig. 3 runs at a slightly higher molecular weight than the COS-expressed ST and the protein detected in rat liver cytosol, this being due the presence of the 6 × His 'tail'. Antibodies specific to particular forms of ST proteins could also be prepared using the above described system by expressing tagged peptide fragments that are unique to the protein of interest, Clearly, the *E. coli* expression and purification system is an extremely versatile tool for preparing antigens for antibody generation, and we are currently using this approach with our human ST cDNAs to develop tools to investigate the tissue expression and distribution of these encoded proteins.

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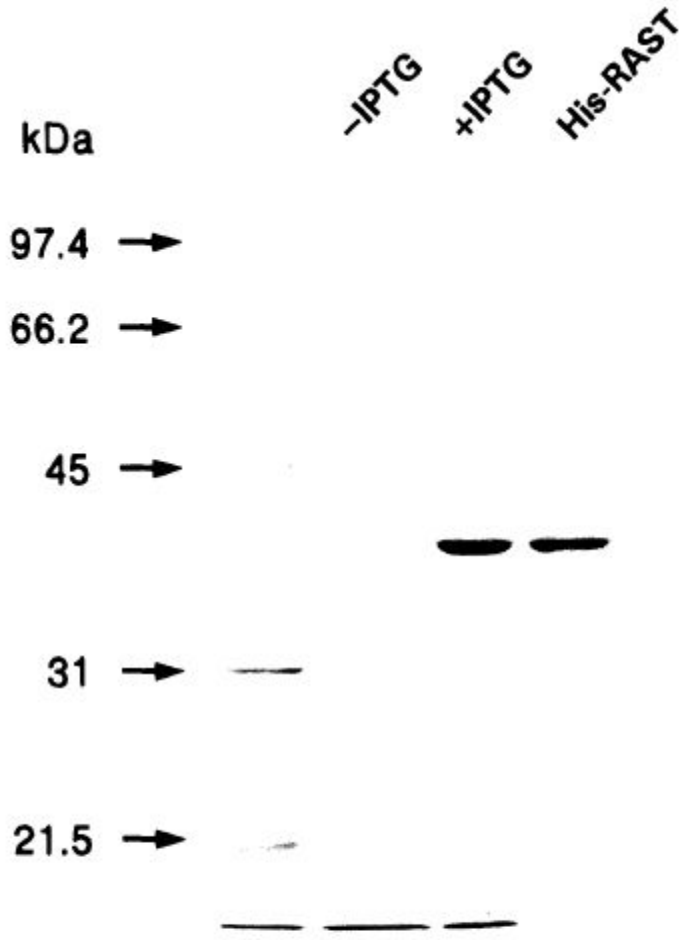

Fig. 2. Analysis of purified rat aryl ST in *E. coli*. Samples were analysed by electrophoresis on 10% SDS-polyacrylamide gels stained with Coomassie blue. Lane 1, molecular weight standards (Bio Rad); Lane 2, 20 μg of total cell lystae protein from *E. coli* transformed with the rat aryl ST cDNA/pQE11 construct before isopropyl-β-D-thiogalactopyranoside (IPTG) induction; Lane 3, same as lane 2 but after induction with IPTG; Lane 4, 3 μg of purified 6 × His tagged rat aryl ST (His-RAST). Briefly, an overnight culture of SG13009[pREP4] *E. coli* cells transformed with the rat aryl ST cDNA in the pQE11 expression vector were used to innoculate 250 ml of media which were grown to mid-log phase with shaking at 37°C before inducing expression of ST protein by the addition of IPTG to a final concentration of 2 mM and allowing the cells to grow for a further 3 h. Following a centrifugation step the pelleted cells were solubilised in a 6 M guanidine HCl buffer (pH 8.0) containing 0.1 M Na_2PO_4 and 0.01 M Tris HCl, and the cleared lysate loaded onto a column containing 2 ml of Ni-NTA resin. After *E. coli* proteins were washed away using the above buffer followed by a 8 M urea buffer (pH 6.3) also containing the salts as the previous buffer, the 6 × His tagged rat ST protein was eluted with this latter buffer containing 250 mM imidazole (pH 6.3).

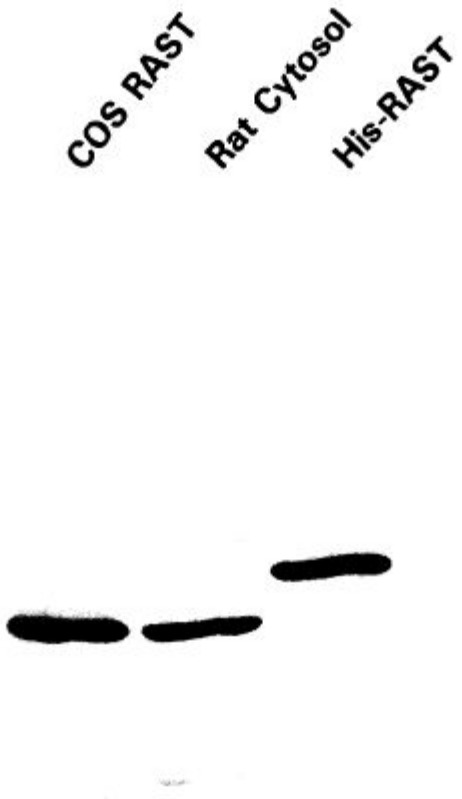

Fig. 3. Western immunoblot analysis of COS- and *E. coli*-expressed rat aryl ST and rat liver cytosol. Lane 1, 2 μg of total cell protein of COS-expressed rat aryl ST (COS RAST); Lane 2, 10 μg of rat liver cytosol; Lane 3, 300 ng of purified 6 × His tagged rat aryl ST (His-RAST). Proteins were subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. This was probed with an antibody raised in the rabbit to the recombinant 6 × His tagged rat aryl ST purified from *E. coli*, as described in Fig. 2, and visualised using TMB stabilised substrate (Promega, Madison, WI, USA).

We were also particularly interested in whether *E. coli* expression may also provide an alternative to the COS celt system for undertaking functional analyses, considering the levels of expression are markedly higher in *E. coli.* Unfortunately, as mentioned above, during investigation of the expression of the ST protein in the above 'QIAexpress' system, the protein was present in *E. coli* as insoluble inclusion bodies, as evidenced by SDS-PAGE analysis (data not shown). This required the use of strong denaturing agents to solubilise the protein, affecting the ability to determine enzyme function. We tried measuring catalytic function of rat aryl ST protein in washed whole *E. coli* cells after IPTG induction and did find activity towards phenol sulfation however, the activity was not higher than that obtained for COSexpressed rat ST protein. In addition, background activity was appreciably higher in untransformed *E. coli* cells. Sonication and/or lysozyme treatment of cells (to lyse cells) failed to increase activity. These results suggest that although extensive inclusion body formation exists a small proportion is soluble and hence active. Attempts to decrease inclusion body formation and increase soluble protein by altering induction temperature (30°C vs. 37°C) and strain of *E. coli* (SGI3009[pREP4] versus M15[pREP4]) had little affect on improving activity. Other strains of *E. coli* as well as methods to regenerate (i.e., refold) the solubilised protein are currently under investigation in this laboratory to enable generation of large amounts of functionally active pure

protein. This type of expression system also seems the most likely to be able to generate sufficient amounts of enzyme for undertaking the crystallisation of ST proteins. This will increase enormously our understanding of the structure/function relationships of ST proteins.

In conclusion, we have shown that the COS cell expression system is a suitable heterologous system to study the catalytic function of cloned mammalian ST cDNAs. This is evident from the successful characterisation of a rat and human aryl ST in our laboratories. The use of this eukaryotic expression system also makes possible studies which can give insight into the structural basis of ST substrate specificity. Examples include the use of studies which identify specific amino acids and domains of ST proteins thought to be involved in substrate or cofactor binding by expressing in COS cells altered STs engineered by site-directed mutagenesis or chimeric protein constructs. Although the *E. coli* expression system we employed was not entirely suitable for functional analyses of STs it was found to be extremely useful for the generation of large amounts of ST suitable as antigen for antibody production. The basis of these techniques of heterologous expression now provides us with the means to make significant inroads into the structural and functional characterisation of cloned STs as well as their expression and distribution in a range of tissues.

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