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Sophie Rahuel-Clermont, Franck Daligault, Marie-Helene Piet, Sandrine Gulberti, Patrick Netter, et al.. Biochemical and thermodynamic characterization of mutated β 1,4-galactosyltransferases 7 involved in the progeroid form of the Ehlers-Danlos syndrome. *Biochemical Journal*, 2010, 432 (2), pp.303-311. 10.1042/BJ20100921 . hal-00535585

HAL Id: hal-00535585

<https://hal.science/hal-00535585v1>

Submitted on 12 Nov 2010

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Biochemical and thermodynamic characterization of mutated β 1,4-galactosyltransferases 7 involved in the progeroid form of the Ehlers-Danlos syndrome

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Short Title: mutated β 1,4-GalT7 and Ehlers-Danlos syndrome

Three mutations of the β 4GALT7 gene corresponding to A186D, L206P and R270C were identified in patients with the progeroid form of the Ehlers-Danlos syndrome and described to be associated with reduction or loss of activity of the β 1,4-galactosyltransferase7. But the molecular bases of the reduction or loss of activity remain to be determined. In the present study, wild-type, A186D, L206P and R270C β 1,4-GalT7s were expressed in *CHO618* cells as membrane proteins and in *Escherichia coli* as soluble maltose binding-fusion proteins. Ability of expressed proteins to transfer galactose from donor to acceptor substrate was systematically characterized by kinetic analysis. The physicochemical properties of soluble proteins were explored by isothermal titration calorimetry which is a method of choice to have access to the thermodynamic parameters of the binding of substrates. Altogether, the results showed that (i) the L206P mutation abolished the activity when L206P β 1,4GalT7 was either inserted in the membrane or expressed as a MBP-full length β 1,4GalT7, (ii) the A186D mutation weakly impaired the binding of the donor substrate, (iii) the R270C mutation strongly impaired the binding of the acceptor substrate. Moreover, *ex vivo* consequences of the mutations were investigated by evaluating the priming efficiency of xylosides on glycosaminoglycan chain initiation. The data demonstrate (iv) a quantitative effect on the glycosaminoglycan biosynthesis depending on the mutations. While the GAG biosynthesis was fully inhibited by the L206P mutation and decreased by the R270C mutation, the A186D mutation did not affect the GAG biosynthesis severely.

Key words: β 1,4-galactosyltransferase; Ehlers-Danlos syndrome; mutation; substrate binding; isothermal titration calorimetry; kinetics; glycosaminoglycan

Abbreviations used: PG, proteoglycan ; GAG, glycosaminoglycan ; HP, heparin ; HS, heparan-sulfate ; CS, chondroitin-sulfate ; DS, dermatan-sulfate ; ECM, extracellular matrix; ITC, isothermal titration calorimetry ; GalT, galactosyltransferase ; MBP, maltose-binding protein ; Gal, galactose ; Xyl, xylose ; pNP-Xyl, 4-Nitrophenyl- β -D-xylopyranoside ; 4MU-Xyl, 4-Methylumbelliferyl- β -D-xylopyranoside ; WT, wild-type.

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INTRODUCTION

Ehlers-Danlos syndrome (EDS) is a heterogeneous group of more than 10 inherited connective-tissue disorders characterized by joint hypermobility, cutaneous fragility, and hyperextensibility [1]. All involve a genetic disorder defect in collagen and connective-tissue synthesis and structure. The different types of EDS are classified according to the signs and symptoms that are manifested and each type of EDS is a distinct disorder that “runs true” in a family. To date, among those, only 5 patients have been described with the progeroid variant of EDS, which is a very rare disorder characterized by a premature aging phenotype with a loose, elastic skin, failure to thrive, joint laxity, psychomotor retardation, hypotonia and macrocephaly [2-4]. Biochemically, aberrant glycosaminoglycan (GAG) substitution of proteoglycans (PG) has been observed in those patients [5, 6].

PGs are found predominantly in the extracellular matrix (ECM) or associated with the cell surface of most eukaryotic cells. Most often, PGs act as molecular organizers of the ECM and as promoters of cell adhesion [7]. They also fulfil a variety of other biological functions, such as growth modulation, ionic filtration and biomechanical lubrication [8-11]. Their biological roles are closely related to the presence and the composition of the GAG chains, which contain binding sites for various growth factors, cytokines, morphogens, enzymes and other signalling molecules [12-15]. Decorin is classified as a small PG with a core protein of about 36 kDa monosubstituted by a dermatan sulfate chain, and a defective glycosylation of this PG is involved in the progeroid variant of EDS [5, 6]. Recently, structural alteration of heparan sulfate chains has also been described and associated with the altered wound repair phenotype of EDS patients [16].

GAG chain initiation begins with the formation of a tetrasaccharide linkage region (GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1) bound *via* the xylosyl residue to serines of the core protein [17]. The biosynthesis of this region is catalyzed, step by step, by distinct glycosyltransferases expressed in Golgi. From this linker tetrasaccharide, the sugar chains are extended by addition of two alternating monosaccharides, an aminosugar and a glucuronic acid [18]. The reaction leads to the formation of heparin and heparan-sulfate (HP/HS) when the aminosugar is *N*-acetyl-glucosamine, and to chondroitin-sulfate and dermatan-sulfate (CS/DS) when it is *N*-acetyl-galactosamine. The progeroid variant of EDS is caused by defects in the β 1,4-galactosyltransferase 7 (β 1,4-galT7), which catalyzes the transfer of the first galactose residue to the xylose of the tetrasaccharide linkage region [2, 3]. Patients with the autosomal recessive progeroid variant of EDS present mutations in the coding sequence of the β 4GALT7 gene. To date, from these patients, three mutations of the β 4GALT7 gene corresponding to A186D, L206P and R270C substitutions have been identified [4, 19-21] which were associated with either reduction or loss of activity. However, the structural bases of these changes remain to be elucidated although a three-dimensional structure of the catalytic domain of the β 1,4-GalT7 from *Drosophila* in the presence of UDP has been recently solved [22]. Having access to the kinetic parameters (i.e., k_{cat} and K_m) values brings information but should be associated with access to thermodynamic parameters of substrate binding in order to interpret the consequences of the amino acid substitutions. Recently, we have validated isothermal titration calorimetry (ITC) method as a tool for detecting and quantifying the interactions between the substrates and the binding sites of the wild-type β 1,4-GalT7 [23].

In the present study, mutated β 1,4-GalTs7 were expressed as soluble maltose-binding fusion proteins in which the *N*-terminal cytoplasmic domain, the transmembrane segment and the stem region that precede the catalytic domain and correspond to amino acids 1-81 were deleted. The kinetic parameters and the thermodynamic parameters of all the mutated β 1,4-GalTs7 were determined. The consequences of the mutations were also investigated *ex vivo* by evaluating the priming efficiency of xylosides on glycosaminoglycan chain initiation.

MATERIALS AND METHODS

Cloning and expression of human β 1,4-galactosyltransferases 7 in CHO618 cells [24]

The human β 1,4-GalT7 sequence fused with a c-myc-tag was subcloned into pcDNA3.1 (Invitrogen, Cergy Pontoise, France) as previously described [23]. The pcDNA- β 1,4-GalT7 was then used as a template to construct the expression plasmids pcDNA-A186D, -L206P and -R270C by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

To express membrane proteins, β 1,4-galactosyltransferase7-deficient CHO618 cells (ATCC number CRL2241) [24] were cultured in complete Dubelcco's modified Eagle's medium-F12 (Gibco, Cergy Pontoise, France) supplemented with fetal bovine serum (10 %), glutamine (1mM) and penicillin-streptomycin (1 %). The pcDNA expression plasmids (10 μ g) were transfected into CHO618 cells on 100 mm plates using Exgen 500 (Euromedex, Souffelweyersheim, France) according to the manufacturer's recommendations. Two days after transfection, cells were harvested and resuspended in Hepes-sucrose buffer (5 mM Hepes, 0.25 M sucrose, pH 7.4) and briefly sonicated (1 x 5 s). Protein concentration of the resulting membrane fraction was determined by the method of Bradford [25]. The expression level of the wild type, A186D, L206P, L206A and R270C β 1,4-GalT7myc proteins was evaluated by western blot analysis using anti-myc antibodies (Invitrogen, Cergy Pontoise, France) according to the manufacturer's recommendations. Quantification of β 1,4-GalT7myc proteins was performed by densitometry using a scanner employing NIH Image software.

Cloning, expression and purification of soluble forms of the human MBP- β 1,4-galactosyltransferases 7

Human β 1,4-GalTs7 were expressed as maltose-binding (MBP) fused proteins. To express the entire protein fused with the MBP, the β 1,4-GalT7 sequence was subcloned into the expression vector pMALc2x (New England Biolabs, Saint Quentin en Yvelines, France) and used as a template to construct the pMALc2x-L206P_{aa1} expression plasmid by site-directed mutagenesis, as previously described. To express the soluble catalytic domain of β 1,4-GalT7, a truncated form of the β 1,4-GalT7 DNA sequence in which the nucleotides encoding the first N-terminal 81 amino acids are deleted was subcloned into pMALc2x, as previously described [23] and thus used as a template to construct the expression plasmids pMALc2x-A186D_{aa82}, -L206P_{aa82} and -R270C_{aa82} by site-directed mutagenesis. The expression of soluble fused MBP-proteins in *E. coli* BL21(DE3) cells (Invitrogen, Cergy Pontoise, France) and the purification onto an amylose column were performed as described previously [23]. The concentration of the purified fusion proteins was determined by fluorescence using a 2100 Bioanalyser (Agilent, Massy, France), according to the manufacturer's recommendations.

β 1,4-galactosyltransferase activity

β 1,4-galactosyltransferase activity were determined as described previously [23] in the presence of 15 to 30 μ g of total proteins containing membrane β 1,4-GalT7 (i.e., 0.3 to 0.6 g of total proteins.l⁻¹) or 2 to 10 pmol of purified truncated β 1,4-GalT7 (i.e., 0.04 to 0.2 μ M), and various concentrations of 4MU-Xyl or pNP-Xyl (i.e., 0.02 to 10 mM of 4MU-Xyl or pNP-Xyl in the presence of 10 mM of UDP-Gal) and UDP-Gal (i.e., 0.025 to 20 mM of UDP-Gal in the presence of 10 mM of 4MU-Xyl or pNP-Xyl). The estimation of the kinetic parameters was achieved by the incubation of at least 10 different concentrations of substrate. Incubations were performed in duplicates. Data were fitted by non-linear regression to the Michaelis-Menten model using the curvefitter program of Sigmaplot 9.0Tm.

Reaction products characterisation

The characterization of the purified reaction products obtained after glycosylation of 4MU-Xyl in the presence of saturating concentration of UDP-Gal by MBP-truncated wild-type, A186D, L206P and R270C β 1,4-GalTs7 (i.e., deleted of the amino acids 1-81) was performed by NMR analysis, as described previously [23].

Isothermal titration calorimetry

ITC experiments were carried out as described previously [23]. UDP (0.5 to 1 mM) and pNP-Xyl (40 mM) were used as donor and acceptor substrate analogues, respectively. Ligand solutions were diluted in the final dialysis used for the protein to achieve the expected final concentration (UDP, 0.5 to 1 mM; pNP-Xyl, 40 mM) and, injected (35 injections of 4 to 10 μ l) into the reaction cell containing the degassed purified MBP-truncated-fused proteins (i.e., lacking the *N*-terminal amino acids 1-81) (1.4 ml, 30 to 50 μ M). Thermodynamic parameters of the binding of the ligands to the proteins were obtained from individual experiments with three different batches of purified MBP-proteins and for each batch, determination was performed in duplicate. Thermodynamic parameters *n* (stoichiometry), ΔH (enthalpy change) and K_a (association constant) were obtained by non-linear least-squares fitting of experimental data using the single-site model of the Origin 7.0 software package provided with the instrument. The dissociation constant (K_d), the free energy of binding (ΔG) and entropy change (ΔS) were deduced from the K_a and ΔH values.

Labelling and Isolation of Radioactive GAGs

Metabolic labelling of GAGs with [35 S] was performed using a modification of a previously published procedure [24]. Cells were incubated with or without 4MU-Xyl (0 to 1 μ M) for 9 hours in sulfate-free medium (Gibco, Cergy Pontoise, France) containing 10 μ Ci/mL Na_2 [35 S]SO₄ (Perkin Elmer Life Sciences, Courtaboeuf, France). GAGs were isolated from the medium using a modification of a previously published procedure [26].

Medium was harvested and digested by papain buffer (20 mM sodium phosphate pH 6.8 containing 1 mM ethylenediamine tetraacetic acid, 5 mM *L*-cysteine hydrochloride and 1 g.L⁻¹ papain) for 2 hours at 60°C. After boiling and centrifugation, supernatant was precipitated overnight at 4°C with 3 volumes of potassium acetate (in 5% ethanol). After centrifugation, pellet was resuspended in 0.2 M NaCl and then centrifuged. Supernatant was supplemented by 100 μ g of chondroitin sulfate from shark cartilage (Sigma) and 5% acetylpyridinium chloride (Sigma) and then incubated for 2 hours at 37°C before centrifugation. After washing twice with NaCl (0.2 M, then 2.5 M), GAGs were resuspended in 0.2 M Tris-HCl (pH 8.0) and the radioactivity associated was counted with 4.5 ml of liquid scintillation counting cocktail Ultima-Gold (Perkin Elmer Life Sciences).

RESULTS

β 1,4-Galactosyltransferase activity of the membrane β 1,4-GalTs7

To address the *in vivo* consequences of the mutations on the enzyme function, membrane wild-type, A186D, L206P, L206A and R270C β 1,4-GalTs7 were overexpressed in *CHO618* cells. Overexpression of each recombinant protein in membrane fractions was demonstrated by western blot using anti-myc antibodies. All were expressed with an expected molecular mass of approximately 37 kDa. The expression level of wild-type, A186D, L206P L206A and R270C β 1,4-GalTs7 quantified by densitometry was similar, allowing the determination and the comparison of specific activities at saturating concentrations of 4MU-Xyl and UDP-Gal (referred to as k_{obs} and expressed as nmol of products formed.mg⁻¹ of total protein.min⁻¹).

The ability of each membrane protein to catalyze the transfer of galactose from donor (UDP-Gal) to acceptor (pNP-Xyl) substrate analogues was determined. No activity was detected with the membrane fraction containing the L206P β 1,4-GalT7. The membrane fraction

containing the wild-type β 1,4-GalT7, but also the A186D, L206A and R270C β 1,4-GalTs7 were active. As shown in Table 1, the K_m values of UDP-Gal for membrane A186D and R270C β 1,4-GalTs7 were 8- and 2-fold higher than that for wild type, respectively, whereas no difference was observed for L206A mutant. The K_m values of pNP-Xyl for A186D, L206A and R270C β 1,4-GalTs7 were 4-, 2.6- and 6-fold higher than that for the wild type, respectively. Finally, the k_{obs} values for A186D and R270C β 1,4-GalTs7 were 3- and 1.5-fold lower compared to the wild type, respectively whereas no difference was observed for the L206A mutant.

β 1,4-Galactosyltransferase activity of the MBP-truncated β 1,4-GalTs7

In order to determine the molecular bases of the reduced activity of A186D and R270C β 1,4-GalTs7, and of the loss of activity of L206P β 1,4-GalT7, mutated β 1,4-GalTs7 were expressed and purified as soluble MBP-truncated proteins, as previously performed for the wild-type enzyme [23]. The purified MBP-R270C_{aa82} (Figure 2, lane 1) and MBP-WT_{aa82} (Figure 2, lane 4) migrated as a single band, with an apparent molecular weight of 70 kDa, consistent with the calculated molecular mass of the truncated β 1,4-GalT7 fused with the MBP. Analysis of purified MBP-A186D_{aa82} (Figure 2, lane 3) and MBP-L206P_{aa82} (Figure 2, lane 2) revealed the presence of a major band (~65% and 90 % of purity, respectively) with the same electrophoretic mobility as the MBP-WT_{aa82}, along with several other bands with lower molecular weight. These additional bands were probably due to proteolysis, as they were also recognized by anti-MBP antibodies (data not shown).

As previously shown [23], MBP-WT_{aa82} can transfer the galactose residue from UDP-Gal to β -xylosides with K_m values for UDP-Gal, pNP-Xyl and 4MU-Xyl (i.e., 0.23 mM, 1.27 mM and 0.27 mM, respectively) (Table 2) similar to those of the membrane wild-type β 1,4-GalT7 (i.e., 0.29, 0.85 and 0.24 mM, respectively). MBP-A186D_{aa82} and MBP-R270C_{aa82} were also able to transfer galactose from UDP-Gal to both acceptor substrates, pNP-Xyl and 4MU-Xyl. Structural characterization of the reaction products by NMR analysis showed that MBP-A186D_{aa82} and MBP-R270C_{aa82} formed the same β 1 \rightarrow 4 linkage as MBP-WT_{aa82} between the Gal from UDP-Gal donor substrate and the Xyl from the acceptor substrate in the reaction product (data not shown). The K_m values of UDP-Gal and pNP-Xyl for MBP-A186D_{aa82} were ~9- and 3-fold higher, respectively than those obtained for MBP-WT_{aa82}; for MBP-R270C_{aa82}, K_m values were ~3- and 6-fold higher (Table 2). These increases in K_m values were similar to those observed for the corresponding membrane proteins (for A186D mutant, ~8- and 5-fold higher towards UDP-Gal and pNP-Xyl, respectively; for R270C mutant, ~2 and 6-fold higher). On the other hand, the k_{cat} values measured for the reaction catalyzed by MBP-A186D_{aa82} and MBP-R270C_{aa82} were similar to MBP-WT_{aa82} (Table 2).

MBP-L206P_{aa82}, contrary to membrane L206P β 1,4-GalT7 which displayed no activity, was able to transfer galactose residue from UDP-Gal to acceptor substrate generating the same β 1 \rightarrow 4 linkage product as wild-type β 1,4-GalT7 (data not shown). The K_m values of UDP-Gal and pNP-Xyl were ~3- and 4-fold higher, respectively than those obtained for MBP-WT_{aa82}. The k_{cat} value of MBP-L206P_{aa82} was similar to that of MBP-WT_{aa82} (i.e., 60 min⁻¹) (Table 2). Because of the partial purity of MBP-L206P_{aa82}, the k_{cat} value determined for MBP-L206P_{aa82} was certainly underestimated and rather corresponded to a value of ~100 min⁻¹, assuming a purity of ~65%.

β 1,4-Galactosyltransferase activity of the MBP-WT_{aa1} and MBP-L206P_{aa1} β 1,4-GalT7

To better understand why L206P substitution led to loss of activity for membrane L206P β 1,4-GalT7 while MBP-truncated L206P β 1,4-GalT7 was active, the entire β 1,4-GalT7 (from amino acids 1 to 327) and the corresponding L206P β 1,4-GalT7, both fused with MBP, were produced in *E. coli* and purified, as previously described. SDS-PAGE analysis of purified

MBP-WT_{aa1} (Figure 2, lane 6) and MBP-L206P_{aa1} (Figure 2, lane 5) revealed the presence of a major band with an apparent molecular weight of 85 kDa. As reported previously, many other bands with lower molecular weight and recognized by anti-MBP antibodies were also visible, suggesting a significant proteolysis of these recombinant proteins.

MBP-WT_{aa1} was active, but showed a 40-fold decrease in k_{cat} compared to MBP-WT_{aa82}. (*i.e.*, 1.7 min⁻¹ for MBP-WT_{aa1} versus 66.6 min⁻¹ for MBP-WT_{aa82}) while K_m values of acceptor and donor substrates were increased by a factor of 4 and 3, respectively. The reason why the activity decreases when the *N*-terminal part 1-81 is inserted between MBP and the catalytic domain remains to be determined. Contrary to MBP-WT_{aa1} and MBP-L206P_{aa82}, no detectable activity was observed for MBP-L206P_{aa1} (*i.e.*, $k_{cat} < 0.05$ min⁻¹) (Table 3).

Thermodynamic characterization of the substrate binding to MBP-A186D_{aa82} and MBP-R270C_{aa82}

As shown from the comparison of the K_m values of the acceptor and donor substrates for wild-type and mutant β 1,4-GalTs7, A186D and R270C substitutions lead to increase of the K_m values of both substrates. It is therefore difficult to conclude with certainty whether Ala-186 and Arg-270 contribute to the donor or the acceptor sites, and thus to interpret the consequences of the substitutions at the structural level. Consequently, the thermodynamic parameters of the binding of donor and acceptor substrates to MBP-A186D_{aa82} and MBP-R270C_{aa82} comparatively to MBP-WT_{aa82} were explored by ITC. Exothermic binding profiles were obtained with all the purified proteins. Typical calorimetric profiles of the binding substrates analogues to MBP-WT_{aa82} and MBP-A186D_{aa82} are presented in Figure 3. Similar profiles were obtained for the MBP-R270C_{aa82}.

Whatever the mutated enzyme, the stoichiometry of UDP binding was comprised between 0.4 to 0.6, due to the possible dimeric state of the enzyme, as described previously [23]. The binding of donor substrate analogue to wild-type, A186D and R270C β 1,4-GalTs7 was driven by an enthalpy change, which was partially counterbalanced by an unfavourable entropy change (Table 4). Binding of UDP to MBP-R270C_{aa82} showed similar thermodynamic parameters as MBP-WT_{aa82}, suggesting no modification of UDP binding by the R270C substitution (Table 4). On the contrary, binding of UDP to the enzyme was significantly modified by the substitution of Ala 186 residue by Asp (Figure 3A), with a significant decrease of ΔH that was not fully offset by a decrease in ΔS . Therefore, dissociation constant was 4-fold higher to that of MBP-WT_{aa82} (Table 4).

Similarly, the consequences of the A186D and R270C substitutions for the binding of acceptor substrate (pNP-Xyl) to the β 1,4-GalT7 were analyzed by ITC (Figure 3B). The pNP-Xyl substrate was used because of the limited solubility of 4MU-Xyl. Because UDP is required to promote the binding of the acceptor substrate to the enzyme, we explored the binding of pNP-Xyl in the presence of UDP and Mn²⁺, as previously described [23]. Since the K_d value obtained for the binding of pNP-Xyl to the wild type was higher than the protein concentration, the *c*-value ($c = [\text{Prot}]_t / K_d$) was lower than 1. Under low *c*-value conditions, both ΔG and ΔH can be measured with confidence provided that, at least, 80% saturation is achieved and that the stoichiometry *n* is known, as described by Turnbull and Danaras [27]. For MBP-WT_{aa82} and MBP-A186D_{aa82}, but not for MBP-R270_{aa82}, it was possible to reach saturating ligand concentration. Therefore, we used the stoichiometry value determined independently by the active site titration experiments with UDP under higher *c*-values conditions, for fitting the low *c*-value titrations. Consequently, a dissociation constant of 4.6 mM was measured for the binding of pNP-Xyl to MBP-WT_{aa82} (Table 5). Its binding to MBP-WT_{aa82} was driven by an enthalpy change (-11.8 kcal/mol) with an unfavourable entropy change. Binding of pNP-Xyl to MBP-A186D_{aa82} was relatively similar to that of MBP-WT_{aa82}, with an estimated dissociation constant of about 5.8 mM (Figure 3B). The magnitude

of ΔH was higher (by about 13 kcal/mol) than that obtained for MBP-WT_{aa82}, yet fully offset by an equivalent increase in entropy, resulting in a similar affinity as the wild type. Contrary to MBP-A186D_{aa82}, the binding of pNP-Xyl to MBP-R270C_{aa82} was difficult to detect by ITC and the saturation was not reached, preventing an accurate determination of the thermodynamic parameters. Nevertheless, the estimated binding affinity seemed to be very weak ($K_d > 10$ mM).

Stimulation of the β 1,4-GalT7-dependent GAG synthesis by glycosides

To identify the consequence of the mutations on the GAG synthesis, we overexpressed membrane wild-type, A186D, L206P and R270C β 1,4-GalTs7 in *CHO618* cells and evaluated the GAG synthesis level after transfection. Immunoblot analysis showed that all β 1,4-GalTs7 were produced at similar level (as shown in Figure 1). Analysis of the GAG synthesis rate 48h after transfection led to a 4-fold increase of ³⁵S-incorporation compared to mock-transfected cells (Figure 4), indicating that overexpression of β 1,4-GalT7 significantly enhanced GAG synthesis. Surprisingly, overexpression of A186D or R270C β 1,4-GalT7 stimulated GAG synthesis at the same level as wild-type β 1,4-GalT7. Only L206P β 1,4-GalT7 failed to increase GAG synthesis, with a rate similar to that of mock-transfected cells (Figure 4).

To further understand the consequences of the A186D and R270C substitutions on GAG synthesis, we investigated the ability of 4MU-Xyl to prime the β 1,4-GalT7-dependent GAG synthesis in *CHO618* cells after transfection by pcDNA- β 1,4-GalT7s. Two days after transfection, cells were incubated for 9 hours with 4MU-Xyl, and the ability of glycoside to prime GAG synthesis in transfected cells was evaluated. Kinetics of GAG synthesis stimulation by 4MU-Xyl (from 0 to 1 μ M) in *CHO618* cells transfected by pcDNA- β 1,4-GalT7s are presented in Figure 5. Stimulation of GAG synthesis was not observed in *CHO618* cells transfected by pcDNA-L206P, whatever the 4MU-Xyl concentration. In *CHO618* cells transfected by pcDNA- β 1,4-GalT7, -A186D and -R270C, GAG synthesis stimulation was a linear function of 4MU-Xyl concentration. However, the GAG synthesis was less stimulated in *CHO618* cells transfected by pcDNA-A186D and -R270C. Therefore, we determined the slope of the linear regression which was representative of the GAG synthesis stimulation. In cells transfected with pcDNA- β 1,4-GalT7, the slope was 0.04. In cells transfected with pcDNA-A186D and -R270C, slopes markedly decreased to 0.03 and 0.02, respectively.

DISCUSSION

β 1,4-GalT7 catalyses a limiting step in the biosynthesis of GAG chains [28, 29]. Patients with mutation in β 4GALT7 gene suffer from skin, cartilage and bone alterations [2]. Only three mutations of the β 4GALT7 gene (A186D, L206P and R270C) have been so far identified [4, 19-21], but leading only to two different genotypes (i.e., compound heterozygosity for the A186D and L206P ; homozygosity for the R270C) [4, 19-20]. Surprisingly, whatever the genotype, patients with the progeroid variant of EDS showed the same premature aging phenotype. The aim of this work was to elucidate the biochemical and structural consequences of the β 4GALT7 mutations on the enzyme function using ITC as a method of choice for probing interactions at the substrate-binding sites of the enzyme [23, 30-32].

Because the wild-type β 1,4-GalT7 catalytic domain (from amino acid 82 to the end of the protein) fused with the MBP is highly soluble and shows K_m values identical to those of the membrane wild-type β 1,4-GalT7 [23], we decided to express in the current study the catalytic domain of the mutated proteins fused with MBP. However, before exploring the physicochemical properties of the MBP-fused truncated enzymes, we systematically

compared by kinetic analysis their ability to transfer the galactose residue from UDP-Gal to acceptor substrate analogue to that of the corresponding enzyme expressed in Golgi membranes of eucaryotic cells.

The MBP-A186D_{aa82} is able to transfer galactose residue from UDP-Gal to acceptor substrate by generating the same $\beta 1 \rightarrow 4$ linkage product as wild-type $\beta 1,4$ -GalT7 (shown as in reference 23). However, the catalytic efficiencies of MBP-A186D_{aa82} with UDP-Gal donor and 4MU-Xyl and pNP-Xyl acceptors are 10, 10 and 4-fold lower, respectively, than those of MBP-WT_{aa82}, due to higher K_m values. Almeida *et al* [19] also reported an increase of the K_m value for the donor substrate (4 fold higher), whereas no significant change was observed for the binding of the acceptor substrate. The ITC results clearly demonstrate that MBP-A186D_{aa82} was able to bind donor and acceptor substrate analogues. But, the K_d value of UDP for MBP-A186D_{aa82} was 4-fold higher than that of wild type, while that of pNP-Xyl was similar to the wild type. These results suggest that Ala 186 contributes directly or, more probably, indirectly to the binding donor site [22]. Interestingly, the ΔH for the binding of UDP to MBP-A186D_{aa82} was significantly reduced by about 15 kcal.mol⁻¹ compared to wild type. In our recent study, a major contribution of the β -phosphate, and more moderately the α -phosphate of the donor substrate to the binding to $\beta 1,4$ -GalT7 was demonstrated [23]. Therefore, the carboxyl side chain of Asp-186 in MBP-A186D_{aa82} could induce repulsive interactions with the phosphate groups of UDP, and prevents the formation of a network of interactions between the donor substrate analogue and the protein. However, Ala-186 which is located in the hydrophobic core of the enzyme and surrounded by bulky aromatic residues [22, 33] likely is not essential in binding UDP in the wild type. This is also in accordance with the fact that Ala-186 corresponds to Ser-274 in $\beta 1,4$ -GalT1, an uncharged hydrophilic residue, capable of forming hydrogen bonds instead of hydrophobic [19].

Concerning the R270C substitution, few biochemical information is currently available. This substitution was previously reported to decrease by approximately 3-fold the $\beta 1,4$ -galactosyltransferase activity in $\beta 1,4$ -GalT7 R270C fibroblasts compared to control fibroblasts [6]. Herein, MBP-R270C_{aa82} was active, but the k_{cat}/K_m ratio determined towards 4MU-Xyl was particularly affected (11-fold lower compared to wild type) due to higher K_m value, whereas that towards UDP-Gal was almost similar to the wild type. The ITC results clearly demonstrate that Arg-270 does not interact directly with the donor substrate analogue, as MBP-R270C_{aa82} was unaffected for UDP binding. On the contrary, the binding of acceptor substrate analogue to MBP-R270C_{aa82} was particularly modified and the binding isotherm did not reach saturation, preventing accurate determination of thermodynamic parameters of binding. The drastic loss of pNP-Xyl binding by MBP-R270C_{aa82} suggests that this positively charged residue is critical for acceptor substrate binding. In the absence of a 3D-structure of the complex $\beta 1,4$ -GalT7/acceptor substrate, it is difficult to explain the requirement of Arg-270 residue in the binding of acceptor substrate. Sequence comparison of human $\beta 1,4$ -galactosyltransferase family members showed sequence variations in their oligosaccharide binding site, which would account for their respective preferences for different oligosaccharides [34, 35]. We suggest that Arg-270 residue which is a lysine residue in the *Drosophila* enzyme [22], and is located on a flexible loop, may interact directly with acceptor substrate by salt bridge or hydrogen bonding interactions or may be a structural element in the organization of the acceptor active site.

Substitution of Leu-206 by Pro was previously reported to inactivate the enzyme [19, 20]. In these studies, L206P $\beta 1,4$ -GalT7 was expressed either as a truncated protein lacking the first *N*-terminal 53 amino acids fused to protein A [20] or as a secreted form lacking the first *N*-terminal 63 amino-acids. Similar results were found in our study when the full length L206P protein was expressed either as a membrane protein or as a soluble protein fused with the MBP. As shown from the amino-acid alignment, Leu-206 is conserved in all mammalian

β 1,4-GalTs7 and in the related *Caenorhabditis Elegans* sqv-3 [36], while it is a valine residue in the *Drosophila* enzyme [22]. The Leu-206 or its equivalent Val is located in the hydrophobic core of the protein [22, 37]. Therefore, due to the location of Leu-206 and the fact that the L206A mutant shows kinetic parameters similar to the wild type, Leu-206 has probably no role in the catalytic efficiency of the enzyme. As suggested by Almeida *et al.* [19] and Okajima *et al.* [20], Pro substitution at position 206 could drastically change the structure of the protein, and therefore would be responsible for the loss of activity. In our study, however, when L206P β 1,4-GalT7 was expressed as a fusion MBP protein, deleted of the first *N*-terminal 81 amino acids, a catalytic activity as efficient as that of its wild-type counterpart was observed. Such a result indicates that substitution of Leu-206 by Pro does not modify the fold of the catalytic domain, at least under a β 1,4-GalT7 form in which the *N*-terminal cytoplasmic domain, the transmembrane segment and the stem region which precedes the catalytic domain have been deleted. Therefore, our results, combined with those of Okajima *et al.* which showed that L206P β 1,4-GalT7 fused to protein A and in which only the stem is present, is inactive [20] suggest that it is the combination of both the Pro residue at position 206 and at least the stem which leads to loss of activity due likely to a modification of the conformation of the protein and in return of the active site.

Similar clinical symptoms and aberrant GAG substitution of PGs were observed in patients with compound heterozygosity for the A186D and L206P and patients with homozygosity for the R270C [5, 6, 16, 19]. Nevertheless, molecular consequences of the three reported substitutions of the β 1,4-GalT7 identified in EDS patients on the enzyme function are clearly different. L206P β 1,4-GalT7 is inactive due to altered conformation or altered substrates binding, and overexpression of L206P β 1,4-GalT7 in *CHO618* cells is unable to synthesise GAG chains. In contrast, A186D and R270C β 1,4-GalT7s display activity. However, A186D and R270C substitutions lead to a decrease of the β 1,4-GalT7 efficiency due to a lowered binding of donor substrate and acceptor substrate, respectively. In our study, the measurement of the ^{35}S incorporation into GAG, reflecting their biosynthesis rate, was surprisingly similar in wild-type, A186D or R270C β 1,4-GalT7s-transfected *CHO618* cells. Therefore, evaluation of the ability of each mutated protein to prime *ex vivo* the GAG biosynthesis was carried out. Since the requirement for xylosylated core proteins can be bypassed by synthetic β -D-xylosides containing hydrophobic aglycones [38-40], we used 4MU-Xyl as a primer for GAG formation. Priming by β -D-xylosides depended linearly on 4MU-Xyl concentration, suggesting that the acceptor substrate (*i.e.*, the xylosylated-core protein) is a limiting factor of the GAG synthesis. In wild type-transfected cells, stimulation of GAG synthesis was 10-fold higher when 1 μM of 4MU-Xyl was used compared to the condition without glycoside; it was 9- and 6-fold, in A186D- and R270C-transfected cells, respectively. The ability of A186D β 1,4-GalT7 to prime GAG synthesis was thus weakly affected compared to that of wild-type enzyme. If we consider that the availability of acceptor substrate is a limiting factor of GAG synthesis, this result is consistent with the ability of A186D β 1,4-GalT7 to bind the acceptor substrate. *CHO618* cells stably expressing A186D β 1,4-GalT7 were also described to be able of substituting human decorin with GAG chains, however with a considerably decreased efficiency [5]. Because of the limited consequences of the A186D substitution on the GAG synthesis, it would be interesting to know the phenotype of homozygous for A186D in that respect. Only the phenotype of individuals with compound heterozygosity for A186D and L206P is known. Because L206P β 1,4-GalT7 is inactive, this phenotype is certainly essentially associated to the L206P mutation of the β 1,4-GalT7. Moreover, it is likely that homozygosity for L206P is not a viable genotype. Indeed, the transfer of the first galactose on the tetrasaccharide linker region of the GAG chains is strictly catalyzed by the β 1,4-GalT7 and cannot be bypassed by another glycosyltransferase [24]. Contrary to A186D β 1,4-GalT7, the ability of R270C β 1,4-GalT7 to prime GAG synthesis

was largely affected compared to that of wild-type enzyme. This lower priming efficacy of 4MU-Xyl in the R270C β 1,4-GalT7 transfected *CHO618* cells may be due to the substitution of Arg-270 residue by Cys that modifies the binding affinity of the acceptor substrate. The reduced ability of the R270C β 1,4-GalT7 to prime GAG synthesis can explain the defective glycosylation of decorin and biglycan in homozygotic individuals for R270C [6, 16]. On the other hand, in β 1,4-GalT7 R270C fibroblasts, a reduced epimerization of chondroitin-/dermatan sulfate GAG chains [6] and a modified pattern of heparan sulphate chains [16] was observed. Thus, there is evidence that the β 1,4-GalT7 genetic defects, along with a decrease in the GAG biosynthesis rate, also affect the quality and diversity of the chains, *via* epimerization and sulfation reactions, and finally the biological function of these substances.

FOOTNOTES

This work was supported by grants from Agence Nationale pour la Recherche (NT05-3_42251). Grants from Region Lorraine, from Communauté Urbaine du Grand Nancy and from Conseil Général de Meurthe et Moselle were also gratefully acknowledged. ITC experiments on the VP-ITC apparatus were conducted in the "Service Commun de Biophysicochimie des Interactions Moléculaires" of the University Henri Poincaré, Nancy Université.

We would like to give our thanks to A. Kriznik (UMR CNRS-Nancy Université 7214) and O. Fabre (UMR CNRS-INPL 7568) for ITC and NMR technical support, respectively.

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TABLES

Table 1 Apparent kinetic parameters of the membrane wild type and mutated β 1,4-GalTs7 expressed in CHO618 cells towards donor (i.e., UDP-Gal) and acceptor substrates (i.e., pNP-Xyl)

To determine the β 1,4-galactosyltransferase activity, standard reactions were performed in 100 mM cacodylate buffer (pH 7.0) containing 10 mM MnCl_2 and 0.3 to 0.6 g.l^{-1} of total proteins containing membrane wild type or mutant β 1,4-GalT7. The k_{obs} values determined at saturating concentration of both substrates were evaluated after normalization of the β 1,4-GalT7 expression level by immunoquantification by western blotting as described in Experimental procedures. Each data point represents the mean \pm 2 SD of three individual determinations. nd, not determined. * $p < 0.05$ compared to β 1,4-GalT7. nd, not determined; —, activity not detected.

	UDP-Gal			pNP-Xyl	
	k_{obs} ($\text{nmol.min}^{-1}.\text{mg prot tor}^{-1}$)	K_{m} (mM)	$k_{\text{obs}}/K_{\text{m}}$ ($\mu\text{l.min}^{-1}.\text{mg prot tor}^{-1}$)	K_{m} (mM)	$k_{\text{obs}}/K_{\text{m}}$ ($\mu\text{l.min}^{-1}.\text{mg prot tor}^{-1}$)
β 1,4-GalT7	8.0 ± 1.5	0.29 ± 0.01	27.6	0.85 ± 0.07	9.4
A186D	2.5 ± 0.2	$2.41 \pm 0.48^*$	1.0	$3.76 \pm 0.57^*$	0.7
L206P	—	—	—	—	—
L206A	7.7 ± 0.6	0.31 ± 0.03	24.8	$2.29 \pm 0.01^*$	3.4
R270C	5.5 ± 0.9	$0.49 \pm 0.12^*$	11.2	$5.01 \pm 0.11^*$	1.1

Table 2 Kinetic parameters of purified MBP-A186D_{aa82}, MBP-L206P_{aa82} and MBP-R270C_{aa82} towards donor substrate (i.e., UDP-Gal) and acceptor substrates (i.e., 4MU-Xyl and pNP-Xyl)

Standard reactions were performed in 100 mM cacodylate buffer (pH 7.0) containing 10 mM MnCl₂ and 0.04 to 0.2 μM of purified truncated MBP-proteins. Each data point represents the mean ± 2 SD of three or more individual determinations. * p < 0.05 compared to the results obtained with MBP-WT_{aa82}. Because the purities of the MBP-A186D_{aa82} and MBP-L206P_{aa82} can be estimated to be 90 and 65%, the *k_{cat}* values are underestimated and rather correspond to ~ 66 and 100 min⁻¹, respectively.

	Donor substrate			Acceptor substrate			
	UDP-Gal			4MU-Xyl		pNP-Xyl	
	<i>k_{cat}</i> (min ⁻¹)	<i>K_m</i> (mM)	<i>k_{cat}</i> / <i>K_m</i> (min ⁻¹ .mM ⁻¹)	<i>K_m</i> (mM)	<i>k_{cat}</i> / <i>K_m</i> (min ⁻¹ .mM ⁻¹)	<i>K_m</i> (mM)	<i>k_{cat}</i> / <i>K_m</i> (min ⁻¹ .mM ⁻¹)
WT _{aa82}	66.6 ± 1.2	0.23 ± 0.07	289	0.27 ± 0.06	247	1.27 ± 0.07	79
A186D _{aa82}	60.0 ± 3.6	2.07 ± 0.67*	29	2.26 ± 0.80*	26	4.25 ± 0.30*	18
L206P _{aa82}	63.6 ± 1.8	0.65 ± 0.18*	98	2.99 ± 0.61*	21	5.54 ± 0.60*	13
R270C _{aa82}	63.0 ± 6.6	0.63 ± 0.17*	100	3.26 ± 0.53*	19	7.93 ± 0.19*	7.8

Table 3 Kinetic parameters of the purified MBP-WT_{aa82}, MBP-WT_{aa1}, MBP-L206P_{aa82} and MBP-L206P_{aa1} expressed in *Escherichia coli* towards UDP-Gal and towards 4MU-Xyl

Standard reactions were performed in 100 mM cacodylate buffer (pH 7.0) containing 10 mM MnCl₂, 0.04 to 1 μM of purified fused proteins. Kinetic parameters for 4MU-Xyl and UDP-Gal were obtained after the addition of increasing amounts of acceptor or donor substrates (0.02 to 10 mM of 4MU-Xyl in the presence of 10 mM of UDP-Gal ; 0.025 to 10 mM of donor substrates in the presence of 10 mM of 4MU-Xyl) to the standard reaction. Each data point represents the mean ± 2 SD of three individual determinations. nd, not determined.

	UDP-Gal			4MU-Xyl	
	k_{cat} (min^{-1})	K_{m} (mM)	$k_{\text{cat}} / K_{\text{m}}$ ($\text{min}^{-1} \cdot \text{mM}^{-1}$)	K_{m} (mM)	$k_{\text{cat}} / K_{\text{m}}$ ($\text{min}^{-1} \cdot \text{mM}^{-1}$)
WT _{aa82}	66.6 ± 1.2	0.23 ± 0.07	289	0.27 ± 0.06	247
WT _{aa1}	1.7 ± 0.06	0.80 ± 0.10	2.13	0.62 ± 0.02	2.74
L206P _{aa82}	63.6 ± 1.8	0.65 ± 0.18	98	2.99 ± 0.61	21
L206P _{aa1}	< 0.05	nd	nd	nd	nd

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Table 4 Thermodynamic parameters for the binding of UDP to the MBP-WT_{aa82}, MBP-A186D_{aa82} and MBP-R270C_{aa82}

ITC experiments were carried out at 30°C in MOPS (20 mM) buffer (pH 7.0) containing NaCl (150 mM) with MnCl₂ (5 mM) using a VP-ITC MicroCalorimeter (Micro Cal, Inc.) Each data point represents the mean ± 2 SD of three individual determinations. * p<0.01 compared to WT.

	ΔG (cal/mol)	ΔH (kcal/mol)	ΔS (cal/mol/K)	$-T\Delta S$ (kcal/mol)	K_d (μM)
WT _{aa82}	- 6880 ± 160	- 22.9 ± 4.8	- 54	16.07	9.0
A186D _{aa82}	- 6030 ± 40	- 8.1 ± 1.3*	- 6.9*	2.08*	38*
R270C _{aa82}	- 6580 ± 70	- 21.0 ± 2.3	- 48.5	14.44	15

Table 5 Thermodynamic parameters for the binding of pNP-Xyl to the MBP-WT_{aa82}, MBP-A186D_{aa82} and MBP-R270C_{aa82}

ITC experiments were carried out at 30°C in MOPS (20 mM) buffer (pH 7.0) containing NaCl (150 mM) with MnCl₂ (5 mM) and UDP (5 mM) using a VP-ITC Microcalorimeter (Micro Cal, Inc.) Each data point represents the mean ± 2 SD of three individual determinations. nd, not determined.

	ΔG (cal/mol)	ΔH (kcal/mol)	ΔS (cal/mol/K)	$-T\Delta S$ (kcal/mol)	K_d (mM)
WT _{aa82}	- 3180 ± 10	- 11.8 ± 0.2	- 29	8.60	4.6
A186D _{aa82}	- 3040 ± 80	- 24.8 ± 5.4	- 73	21.83	5.8
R270C _{aa82}	nd	nd	nd	nd	> 10

FIGURE LEGENDS

Figure 1 Immunoblot analysis of membrane fraction of *CHO618* cells expressing wild type and mutant β 1,4-GalT7.

Membrane protein (10 μ g) containing wild type- (lane 1), A186D- (lane 2), L206P- (lane 3), R270C- (lane 4) and L206A- β 1,4-GalT7 (lane 5) were loaded. The β 1,4-GalT7 proteins were probed with primary antibody directed against the c-myc tag.

Figure 2 Analysis of purified recombinant human wild type and mutant β 1,4-GalT7 fused-proteins by SDS-PAGE.

Human wild type and mutant β 1,4-GalT7 were expressed in *E. coli* as fused-proteins with the maltose-binding protein, and then, purified onto an amylose column, as described in *Experimental procedures*. Purified proteins (2 μ g) MBP-R270C_{aa82} (lane 1), MBP-L206P_{aa82} (lane 2), MBP-A186D_{aa82} (lane 3), MBP-WT_{aa82} (lane 4), MBP-L206P_{aa1} (lane 6) and MBP-WT_{aa1} (lane 7) and protein marker (*i.e.*, 25, 37, 75, 100 and 150 kDa) (lane 5) were analysed by electrophoresis on polyacrylamide gel (10%, w/v) in the presence of SDS and then, stained with Coomassie blue.

Figure 3 Calorimetric profiles of the donor and acceptor substrate analogues binding to MBP-A186D_{aa82}, compared to MBP-WT_{aa82}.

A/ Binding of UDP to MBP-WT_{aa82} and MBP-A186D_{aa82}. The reaction cell contained MBP-WT_{aa82} (1.4 mL, 30 μ M, black line) or MBP-A186D_{aa82} (1.4 mL, 50 μ M, gray line) in 20 mM MOPS buffer containing 150 mM NaCl and 5 mM of MnCl₂. The syringe contained 0.5 mM and 1 mM UDP in the same buffer, for MBP-WT_{aa82} and MBP-A186D_{aa82}, respectively. *Top*, raw calorimetric data obtained from the injection of UDP at 4-min intervals. The bottom plot shows the integrated binding isotherm with the experimental points and the corresponding best fit, providing values for thermodynamic parameters. *B/ Binding of pNP-Xyl to MBP-WT_{aa82} and MBP-A186D_{aa82}.* The reaction cell contained MBP-WT_{aa82} (1.4 mL, 30 μ M, black line) or MBP-A186D_{aa82} (1.4 mL, 25 μ M, gray line) in 20 mM MOPS buffer containing 150 mM NaCl containing 5 mM of MnCl₂ and 5 mM UDP. The syringe contained 40 mM pNP-Xyl in the same buffer. *Top*, raw calorimetric data obtained from the injection of pNP-Xyl at 4-min intervals. The bottom plot shows the integrated binding isotherm with the experimental points and the corresponding best fit, providing values for thermodynamic parameters.

Figure 4 Restoration of GAG synthesis in *CHO618* cells after wild type or mutant β 1,4-GalT7 transfection.

CHO618 cells were transfected with pcDNA- β 1,4-GalT7 or -A186D or -L206P or -R270C, then labeled, two days after transfection, with Na₂[³⁵S]SO₄ (10 μ Ci/mL) for 9 h. The labelled ³⁵S-GAGs were isolated by papain digestion and successive acetate potassium, and CPC-precipitations. Respective β 1,4-GalT7 expression was analyzed by western blot using anti-myc antibodies, as described in the *Experimental Procedures*.

Figure 5 Stimulation kinetics of GAG synthesis by 4MU-Xyl in CHO618 cells expressing wild type or mutated β 1,4-GalTs7.

CHO618 cells were transfected with pcDNA- β 1,4-GalT7 or -A186D or -L206P or -R270C, then, two days after transfection, incubated with various concentrations of 4MU-Xyl (0 to 1 μ M) and labeled, in the same time, with $\text{Na}_2[^{35}\text{S}]\text{SO}_4$ (10 μ Ci/mL) for 9 h. The labelled ^{35}S -GAGs were isolated by papain digestion and successive acetate potassium-, and CPC-precipitations.

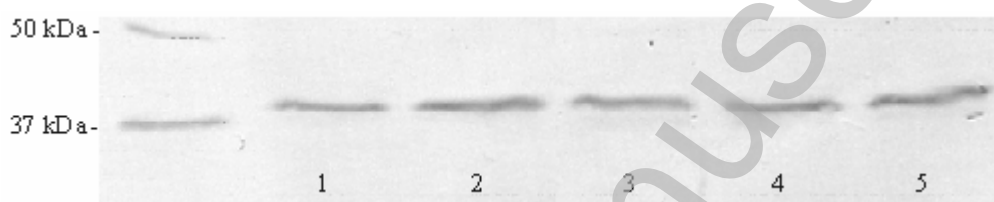


Figure 1



Figure 2

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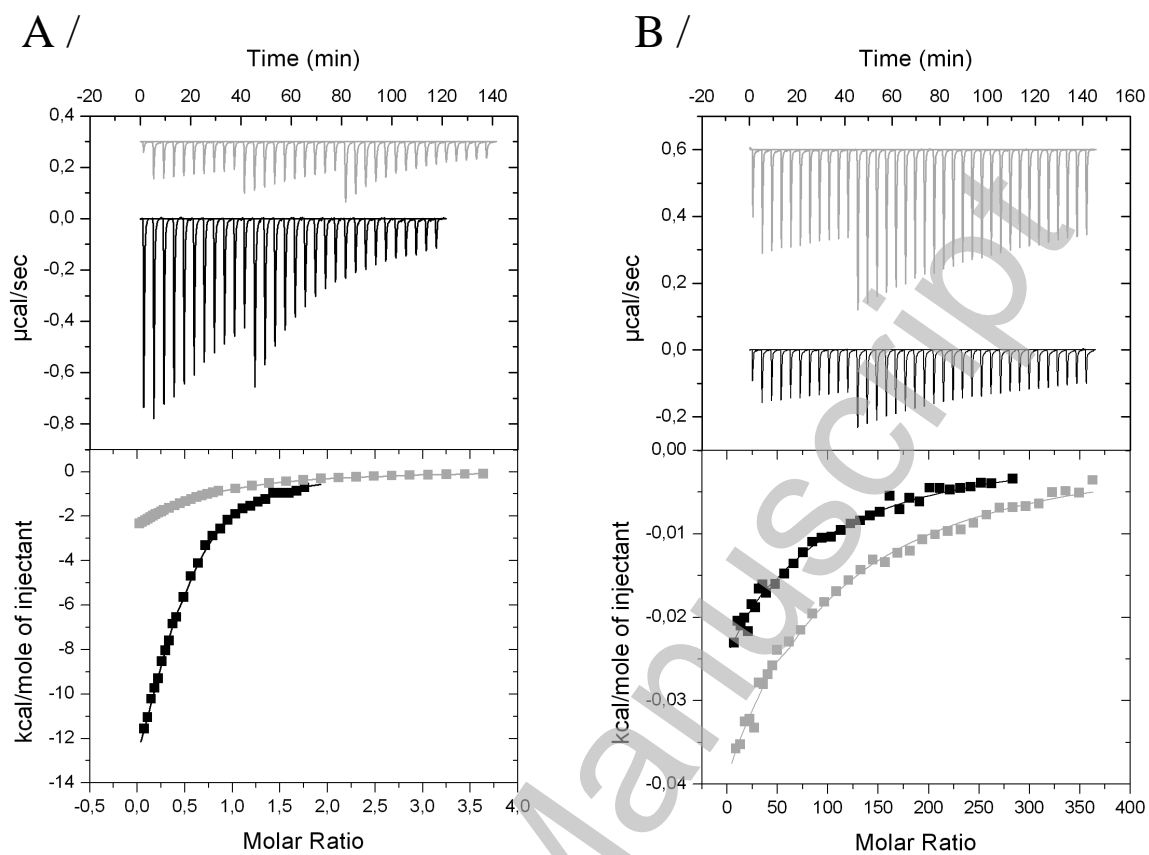


Figure 3

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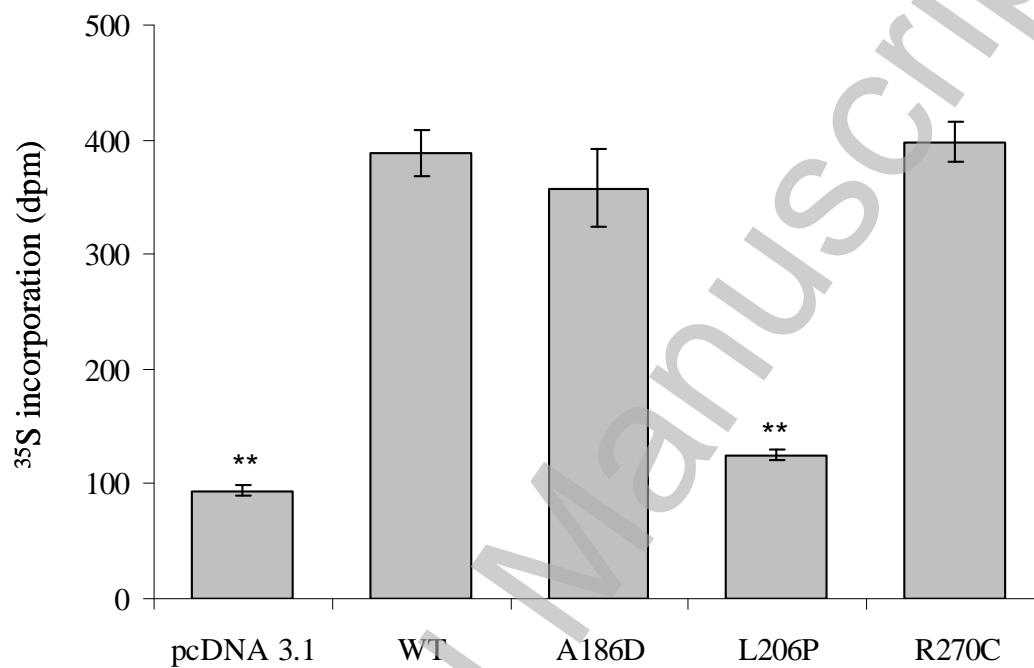


Figure 4

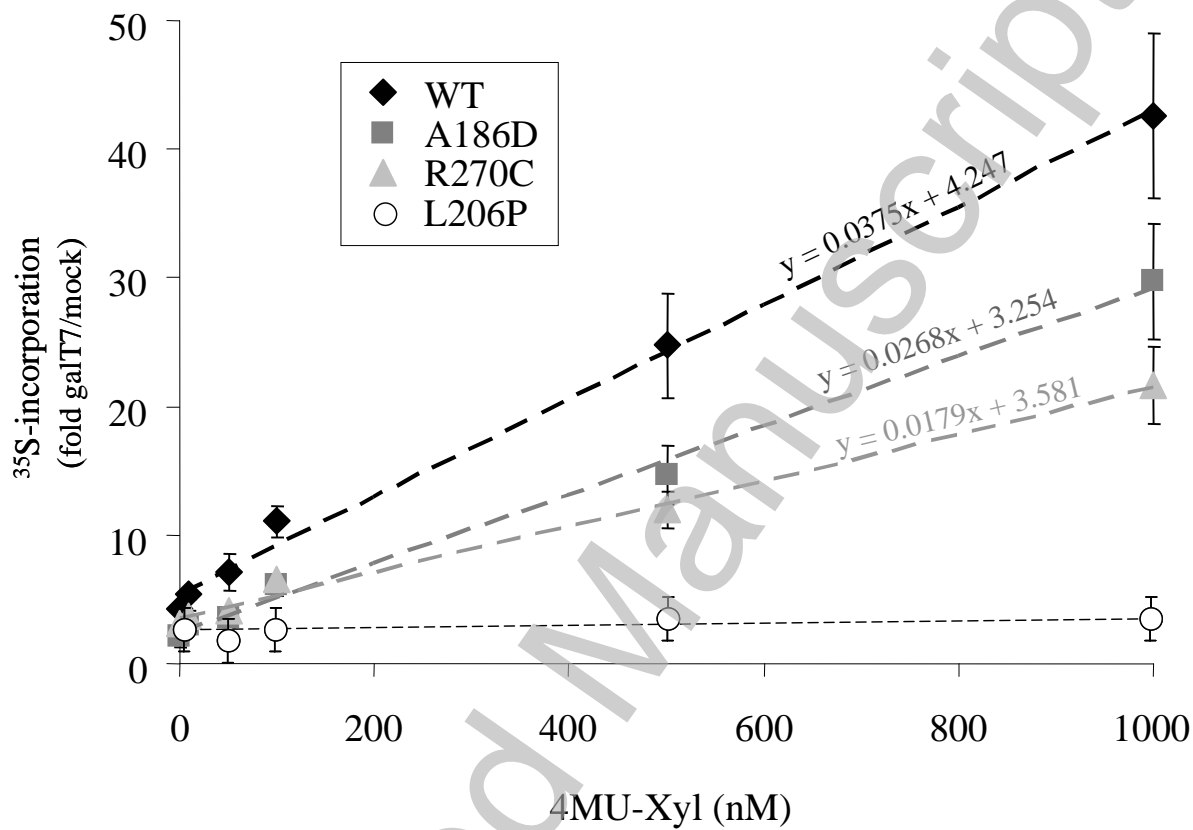


Figure 5

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