O-glycan variability of egg-jelly mucins from *Xenopus laevis*: characterization of four phenotypes that differ by the terminal glycosylation of their mucins

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Eggs from *Xenopus laevis* are surrounded by several layers of jelly that are needed for proper fertilization. Jelly coat is composed of high-molecular-mass glycoconjugates to which are bound many globular proteins. O-glycans released from the jelly coat of *X. laevis* have been partially described in previous studies. In this study, we compared the glycosylation pattern of the egg jelly coat isolated from six specimens of *X. laevis*. The O-glycans were released from jelly coats by alkali/borohydride treatment. Structural characterization was performed through a combination of one- and two-dimensional ^{1}H-NMR and methylation analysis. This allowed the description of a new family of sulphated O-glycans present in jelly coats of all *X. laevis*. However, the jelly O-glycans showed a low extent of polymorphism between specimens. This intra-specific variability was restricted to the terminal substitution of O-linked oligosaccharides. The differential expression of two glycosyltransferase [an α-(1 → 4) galactosyltransferase and an α-(1 → 3) fucosyltransferase] activities resulted in the characterization of four phenotypes of *X. laevis*. Furthermore, electrophoretic analysis suggested that the high-molecular-mass fraction of jelly coat was mostly composed of mucin-type glycoproteins. Blot analysis with lectins confirmed that the glycan variability was borne by these mucin-type components. However, fertilization assays suggested that the glycan polymorphism had no repercussion on egg fertilizability.

Key words: amphibian, fertilization, glycosyltransferase, NMR.

INTRODUCTION

Xenopus laevis eggs are surrounded by a complex extracellular matrix that consists of a vitelline envelope and a thick jelly coat. The jelly coat contains three concentric layers (J1 to J3) which are successively deposited around the egg as it passes through the oviduct [1]. This matrix provides a protective environment for the developing embryo and is involved in many specific interactions such as, sperm binding to the vitelline envelope [2], block to polyspermy [3] and selection of sperm [4].

Carbohydrates themselves are involved in many of these interactions. Indeed, oligosaccharide chains of sperm receptor (gp69/64) seem to be directly involved in the binding function of the protein [2]. Also, the block to polyspermy is in part due to the agglutination of jelly-coat components and a cortical granule lectin (CGL) [3]. The CGL is released from the egg just after sperm penetration and binds to the carbohydrate moieties of two jelly-coat glycoproteins [5]. Thus it is suggested that the ability of sperm to traverse the jelly coat is dependent on interactions with the carbohydrate moieties of egg-jelly components [6].

If jelly coat has been recognized for a long time to be essential for fertilization, little is known about the macromolecules involved in the sperm–egg interaction. The jelly coat is composed of high-molecular-mass glycoproteins that act as a scaffold to which low-molecular-mass proteins, diffusible or not, are bound [7]. However, the respective part these two types of components play in fertilization is still controversial [8,9]. The exact nature of the high-molecular-mass fraction is still unclear. However, the structural and chemical characteristics of the jelly coat suggest that this fraction is mostly constituted by mucin-type glycoproteins. They include: the high content, up to 60 %, of sugar in jelly coat [10], the very high-molecular-mass and the stiff, extended conformation of these constituents [7], the release of high quantities of O-linked mucin-type oligosaccharides after alkaline treatment [11,12] and the absence of uronic acids which are the main constituents of glycosaminoglycans, except keratan sulphates [11]. Furthermore, three mucin-type glycoproteins [frog integumentary mucin (FIM) A1, FIM B1 and FIM C1] have already been described in the *X. laevis* skin [13–15], one of which has the same structure as human mucin MUC 2 [16].

The analysis of *X. laevis* egg-jelly-coat O-glycans identifies 19 different neutral oligosaccharides, classified into two families [11,12]. These glycans appear characteristic of the *X. laevis* species. Indeed, studies of jelly-coat O-glycans from 15 amphibian species demonstrated two main points. First, these components showed a remarkable heterogeneity of structures, more than 300 different O-glycans were identified and approx. 20 new glycosyltransferase activities were demonstrated. Some of these glycans present ubiquitous epitopes such as the Lewis X determinant in jelly coat of the urodele *Axololtl maculatum* [17] or the A-Lewis Y epitope in the urodele *Pleurodeles waltl* [18], while others show non-predictable sequences such as Fuc(α1-5)[Fuc(α1-4)]Kdn(α2-3/6) (where Kdn stands for 3-deoxy-D-glycero-D-galacto-nonulosic acid) found in *Ambystoma tigrinum* [19]. Second, it appeared that these oligosaccharides were highly specific to each amphibian species. Each species analysed showed one to three families of structurally related O-glycans. In most cases, the species specificity is established through the

Abbreviations used: CGL, cortical granule lectin; GC, gas chromatography; GalNAc-ol, *N*-acetylgalactosaminitol; PAS, periodate–Schiff; BSI-B_4, *Bandeiraea simplicifolia* isoform B_4; Hm, Mm and Lm, high-, medium- and low-mobility glycoproteins; FIM, frog integumentary mucin.

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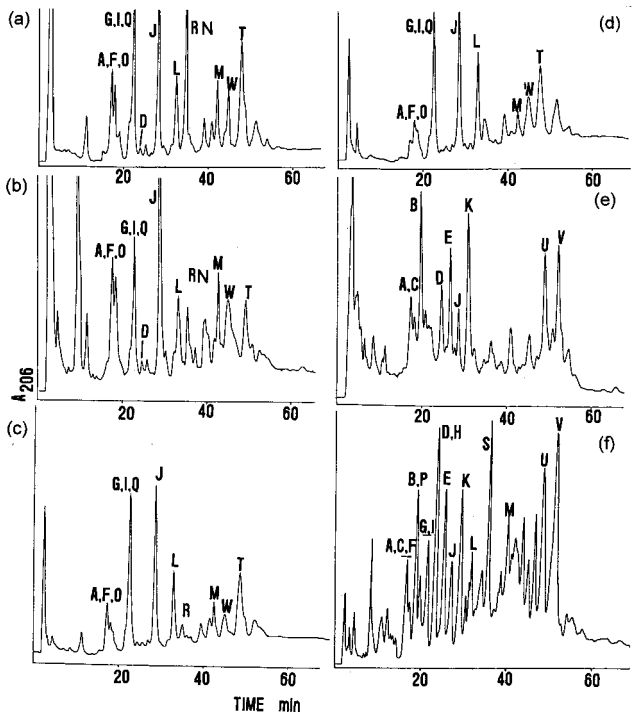


Figure 1 HPLC profiles of oligosaccharide alditols released from the egg jelly of six specimens of *X. laevis* on an amine-bonded silica column

Specimens: 1 (**a**), 2 (**b**), 3 (**c**), 5 (**d**), 4 (**e**) and 6 (**f**). All the designated peaks have been purified and the structure of the corresponding O-glycans analysed. Peaks containing multiple compounds were recycled on a reverse-phase column.

presence of one or a mixture of several original oligosaccharide sequences. *X. laevis* species was characterized by the presence of a blood group A epitope substituted by a GlcNAc residue in an α-(1 $\rightarrow$ 3) linkage on one out of the two O-glycans families, while the other family presents the ubiquitous sequence in amphibians Fuc(α1-2)Gal(β1-3)[Fuc(α1-2)]Gal(β1-3).

The fact that these structural characteristics were found in *X. laevis* from different origins [11,12] is consistent with the hypothesis of a glycan species-specificity. However, such a species specificity is not incompatible with an intra-specific variability such as the 'blood group' polymorphism found in human. If it occurred, such a structural variability of the jelly-coat components might be of importance in the studies of their respective roles during fertilization. Also, if it appeared that this variability was to be of a higher extent than the one observed between species, it would challenge the hypothesis of a species specificity of O-glycosylation. Here, we report that the glycosylation variability of jelly-coat mucins in *X. laevis* is restricted to the terminal substitution of O-glycans and involves two distinct glycosyltransferase activities.

EXPERIMENTAL

Sampling of eggs

Eggs from *X. laevis* were obtained from spawnings induced by injection of 500 units of human chorionic gonadotrophin. Egg-jelly coats from the same clutch were extracted into Dulbecco's phosphate buffered saline (Sigma) containing 10 mM EDTA, 1 mM PMSF and 0.5 % 2-mercaptoethanol at 4 °C overnight. The mixture was centrifuged and the supernatant was then dialysed for 72 h against water and finally freeze dried.

Isolation of oligosaccharide alditols

The material was submitted to alkaline reductive degradation in 100 mM NaOH containing 1 M $NaBH_4$ at 37 °C for 72 h. The reaction was stopped by the addition of DOWEX 50 $\times$ 8 (25–50 mesh, H^+ form) at 4 °C until pH 6.5, and after evaporation to dryness, boric acid was distilled as the methyl ester in the presence of methanol. Total material was submitted to a cationic exchange chromatography on DOWEX 50 $\times$ 2 (200–400 mesh, H^+ form) to remove residual peptides. The oligosaccharide fraction was then purified on a Bio-Gel P2 column (Bio-Rad).

Fractionation of oligosaccharide alditols

Compounds were fractionated by HPLC on a primary amine-bonded silica column (Supelcosyl^{TM}, LC-NH_2, 4.6 mm $\times$ 250 mm, Supelco Inc., Bellefonte, PA, U.S.A.) using a mixture of acetonitrile/30 mM KH_2PO_4/water (75:0:25 to 50:50:0, by vol., in 60 min) with a flow rate of 1 ml/min. Oligosaccharides were detected by UV spectroscopy at 206 nm using an LDC variable-wavelength detector (Spectra Monitor D, Milton Roy, Riviera Beach, FL, U.S.A.) connected to a Spectra-Physics Model 4100 computing integrator. If necessary, fractions were recycled on a 5 μm ODS Zorbax column (4.4 mm $\times$ 250 mm, DuPont Ins., Paris, TX, U.S.A.) using a mixture of water/acetonitrile (99:1, v/v).

PAGE

Dry egg jellies were solubilized in sample buffer (pH 6.8) containing 0.15 M Tris/HCl, 20 % (v/v) glycerol, 4 % (w/v) SDS and 1 % 2-mercaptoethanol [20]. Samples were loaded on to a gradient 2.5–10 % polyacrylamide/SDS gel and run at 5 mA, overnight. For separation of proteins by two-dimensional gel electrophoresis, a mixture of 2.5–4 and 4–6 pH range ampholyte (Biochemika) was used in the first dimension. Gels were stained for carbohydrates using the periodate–Schiff (PAS) method [21]. For Western blotting analysis, proteins were electro-transferred to nitrocellulose sheets and probed with horseradish-peroxidase-labelled *Bandeiraea simplicifolia* isoform B_4 (BSI-B_4) and *Tetragonolobus purpureas* lectins (Sigma) at a dilution of 1:2000. Staining was performed using an ECL® detection kit (Amersham).

Agarose gel electrophoresis

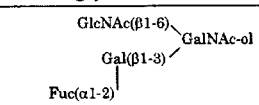
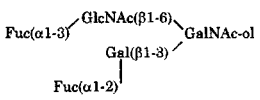
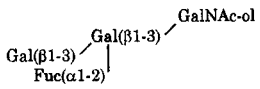
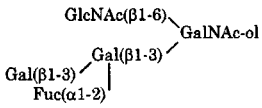
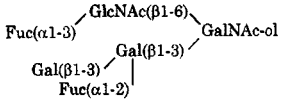
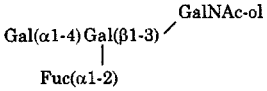
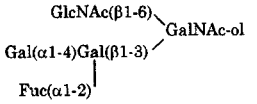
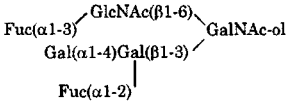
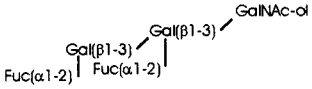
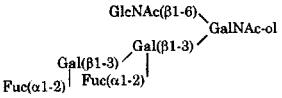
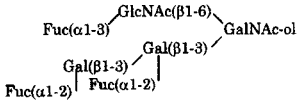
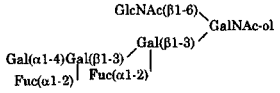
Aliquots of the purified mucins (400 μg) were subjected to agarose gel electrophoresis in veronal buffer, pH 8.2, as described previously [22]. Slides were stained for carbohydrates with PAS reagent and for acidic components with Toluidine Blue.

Enzymic digestions

Samples were dissolved in 200 mM Tris/acetate buffer, pH 7.5, containing 2 mM $CaCl_2$, and the following enzymes were added: hyaluronate lyase from *Streptomyces hyalurolyticus* (type IX, Sigma), chondroitinase ABC from *Proteus vulgaris* (Seikagaku, Tokyo), heparinase III from *Flavobacterium heparinum* (Sigma) in the amounts 100, 50 and 50 m-units/mg of material respectively. The mixture was stirred overnight at 37 °C and

Table 1 Structures of major O-glycans isolated from six specimens of *Xenopus laevis*

Each O-glycan (A to W) is attributed to the specimens from which it has been isolated (+).

O-glycan structures		1	2	3	4	5	6
	A	+	+	+	+	+	+
	B				+		+
	C				+		+
	D	+	+		+		+
	E				+		+
	F	+	+	+		+	+
	G	+	+	+		+	+
	H						+
	I	+	+	+		+	+
	J	+	+	+	+	+	+
	K				+		+
	L	+	+	+		+	+

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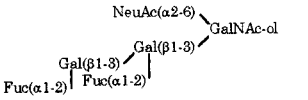
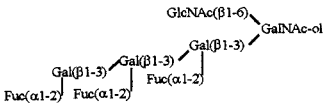
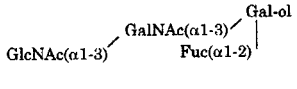
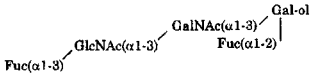
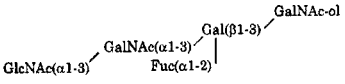
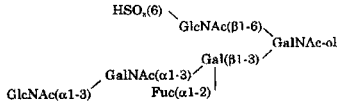
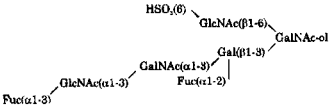
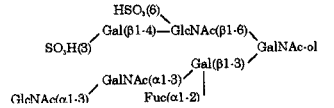
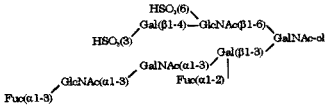
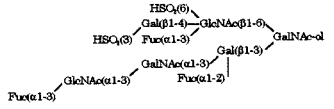
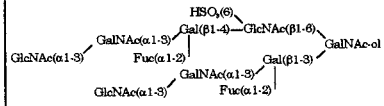
	M	+	+	+		+	+
	N	+	+				
	O	+	+	+		+	
	P						+
	Q	+	+	+		+	+
	R	+	+				
	S						+
	T	+	+	+		+	
	U					+	+
	V					+	+
	W	+	+	+			

freeze dried before being subjected to isopycnic ultracentrifugation.

Alternatively, freeze-dried material was subjected to mild Pronase (Sigma) digestion at 37 °C in 0.01 M calcium acetate buffer, pH 7, at an enzyme ratio of 1:40 (w/w) for 24 h. The glycopeptides were then purified on a Bio-Gel P6 (Bio-Rad) column.

Disulphite reduction and alkylation

Dry material was solubilized in 6 M guanidinium chloride, 0.1 M Tris/HCl, pH 8.0, and reduced with 10 mM dithiothreitol for 5 h at 37 °C. Iodoacetamide was added to a final concentration of 25 mM and left in the dark overnight at room temperature. Reduced and alkylated material was dialysed into SDS/PAGE sample buffer.

Isopycnic ultracentrifugation

Freeze-dried material was dissolved by stirring at 4 °C overnight in Dulbecco's phosphate buffered saline containing 0.02% sodium azide and 42% (w/v) CsBr. Solubilized material was centrifuged at 170000 ***g*** for 72 h at 12 °C, as described previously [23]. Fractions of 1 ml were recovered from each tube and tested

Table 2 ^{1}H NMR chemical shifts of the oligosaccharide alditols A to G

nd, not determined.

		Oligosaccharide alditols, δ (p.p.m.)						
O-Glycan	Reporter group	A	B	C	D	E	F	G
GalNAc-ol I	H-2	4.404	nd	4.337	4.342	4.336	4.404	4.409
	H-3	4.084	4.403	4.108	4.099	4.098	4.109	4.102
	H-4	3.501	4.084	3.574	nd	nd	4.646	3.532
	H-5	4.252	4.252	4.134	nd	4.227	4.128	4.194
	NAc	2.056	2.042	2.051	2.055	2.040	2.046	2.055
Gal(β1-3) II	H-1	4.572	4.570	4.664	4.620	4.620	4.618	4.602
	H-3	nd	nd	4.015	4.009	4.009	nd	nd
	H-4	3.91	3.924	4.228	4.22	4.222	4.043	4.051
	H-6	nd	–	nd	3.93	nd	nd	nd
Gal(β1-3) III	H-1	–	–	4.623	4.640	4.651	–	–
	H-4	–	–	3.925	3.92	4.925	–	–
Gal(α1-4) III/IV	H-1	–	–	–	–	–	4.932	4.937
	H-4	–	–	–	–	–	4.033	4.032
	H-5	–	–	–	–	–	4.326	4.316
GlcNAc(β1-6) II′	H-1	4.551	4.560	–	4.559	4.557	–	4.553
	H-3	nd	nd	–	nd	nd	–	3.546
	H-4	nd	nd	–	3.45	nd	–	3.45
	H-5	nd	nd	–	3.45	nd	–	3.45
	H-6	nd	3.944	–	nd	nd	–	nd
	NAc	2.056	2.054	–	2.055	2.045	–	2.060
Fuc(α1-2) F(II)	H-1	5.222	5.221	5.383	5.361	5.357	5.261	5.231
	H-3	nd	nd	3.892	nd	nd	nd	nd
	H-5	4.275	4.275	4.277	4.25	4.265	4.284	4.282
	H-6	1.244	1.245	2.464	1.232	1.233	1.244	1.245
Fuc(α1-2) F(III)	H-1	–	–	–	–	–	–	–
	H-5	–	–	–	–	–	–	–
	H-6	–	–	–	–	–	–	–
Fuc(α1-3) F(II′)	H-1	–	4.991	–	–	4.989	–	–
	H-5	–	4.327	–	–	4.33	–	–
	H-6	–	1.160	–	–	1.159	–	–

for carbohydrates using the orcinol assay. The high-density fractions ($\rho > 1.4$ g/ml) were pooled and exhaustively dialysed against deionized water and freeze-dried.

400 MHz ^{1}H NMR spectroscopy

NMR experiments were performed on a Bruker ASX 400 WB spectrometer. Chemical shifts are expressed downfield from internal 4,4′-dimethyl-4-silapentane-1-sulphonate but were actually measured by reference to internal acetone ($\delta = 2.225$ in ^2H_2O at 25 °C). The two-dimensional homonuclear COSY were performed using Bruker standard pulse sequences.

Methylation analysis

Permethylation was carried out as described by Ciacanu and Kerek [24]. After methanolysis (0.5 M HCl/methanol), the partially methylated methyl glycosides were peracetylated and analysed by gas chromatography (GC)–MS [25]. The sulphated oligosaccharide alditols were subjected to mild periodate oxidation prior to methylation analysis with sodium meta-periodate in imidazole buffer, at 0 °C for 30 min, as described previously [26]. This specifically cleaved the *N*-acetylhexosaminitol unit, which facilitated the chloroform extraction of unsulphated branchs of the sulphated O-glycans.

Fertilization assay

Assays were done according to Olson and Chandler [8]. Briefly, male testes were removed and macerated in 1.5 × O-R2 buffer (124 mM NaCl, 3.75 mM KCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.5 mM Na_2HPO_4 and 10 mM Hepes). Sperm density was normalized to 5×10^7 sperm/ml. Just ovoposited eggs were covered with F-1 buffer (41.25 mM NaCl, 1.25 mM KCl, 0.25 mM $CaCl_2$, 0.06 mM $MgCl_2$, 0.5 mM Na_2HPO_4 and 2.5 mM Hepes, pH 7.8). Sperm was added to a final concentration of 1.0×10^6 sperm/ml in the fertilization assay medium. Egg development was followed for 6 h after fertilization. Fertilization was scored by counting cleavage stage embryos after 2 h.

RESULTS

Fractionation of oligosaccharide alditols

Egg jellies extracted from individual clutches of six *X. laevis* specimens were subjected to alkaline treatment. Purified O-glycans were sequentially injected on to an amino-bonded silica column, in equivalent conditions. Out of the six resulting chromatographic profiles (Figure 1), four were qualitatively identical (specimens 1, 2, 3 and 5; Figures 1a, 1b, 1c and 1d respectively) while two presented original patterns (specimens 4 and 6; Figures 1e and 1f respectively). This suggested the occurrence of three distinct patterns of glycosylation in the egg jelly of the six specimens. The use of two other chromatographic

Table 3 ^{1}H NMR chemical shifts of the oligosaccharide alditols H to L

nd, not determined.

		Oligosaccharide alditols, δ (p.p.m.)				
O-Glycan	Reporter group	H	I	J	K	L
GalNAc-ol I	H-1	nd	nd	3.81	3.75	3.80
	H-1′	nd	nd	3.76	3.79	3.74
	H-2	4.409	4.24	4.24	4.234	4.24
	H-3	4.099	4.065	4.05	4.044	4.04
	H-4	3.525	4.019	3.62	3.612	3.62
	H-5	4.191	4.089	4.19	4.190	4.19
	H-6	nd	nd	3.92	3.924	3.92
	H-6′	nd	nd	3.70	3.694	3.67
	NAc	2.043	2.046	2.05	2.036	2.05
Gal(β1-3) II	H-1	4.601	4.696	4.68	4.684	4.69
	H-2	nd	nd	3.82	3.813	3.84
	H-3	nd	4.186	4.18	4.178	4.20
	H-4	4.050	4.019	4.02	4.016	4.00
	H-5	nd	nd	nd	nd	3.77
Gal(β1-3) III	H-1	–	4.899	4.89	4.898	5.03
	H-2	–	nd	3.69	3.691	3.72
	H-3	–	nd	3.89	3.895	3.95
	H-4	–	3.906	3.92	3.915	4.05
	H-5	–	nd	nd	nd	3.79
	H-6	–	nd	nd	nd	nd
	H-6′	–	nd	nd	nd	nd
Gal(α1-4) III/IV	H-1	4.936	–	–	–	4.98
	H-2	nd	–	–	–	3.85
	H-3	nd	–	–	–	3.96
	H-4	4.032	–	–	–	4.04
	H-5	4.274	–	–	–	4.38
	H-6	nd	–	–	–	3.71
	H-6′	nd	–	–	–	3.71
GlcNAc(β1-6) II′	H-1	4.563	–	4.56	4.569	4.56
	H-2	nd	–	3.71	3.845	3.71
	H-3	nd	–	3.53	3.641	3.53
	H-4	nd	–	3.44	3.53	3.44
	H-5	nd	–	3.45	3.53	3.45
	H-6	nd	–	3.93	3.930	3.94
	H-6′	nd	–	3.74	3.749	3.74
	NAc	2.056	–	2.05	2.046	2.05
Fuc(α1-2) F(II)	H-1	5.289	5.407	5.41	5.412	5.42
	H-2	nd	nd	3.77	3.767	3.78
	H-3	nd	nd	3.86	3.866	3.86
	H-4	nd	nd	3.81	nd	3.81
	H-5	4.309	4.299	4.27	4.276	4.30
	H-6	1.245	1.230	1.20	1.231	1.19
Fuc(α1-2) F(III)	H-1	–	5.329	5.33	5.332	5.34
	H-2	–	nd	3.80	3.799	3.80
	H-3	–	nd	3.74	3.739	3.71
	H-4	–	nd	3.82	nd	3.81
	H-5	–	4.336	4.33	4.333	4.31
	H-6	–	1.197	1.23	1.197	1.24
Fuc(α1-3) F(II′)	H-1	4.992	–	–	4.984	–
	H-2	nd	–	–	3.694	–
	H-3	nd	–	–	3.830	–
	H-4	nd	–	–	nd	–
	H-5	4.326	–	–	4.334	–
	H-6	1.160	–	–	1.158	–

systems (high performance anion-exchange chromatography and TLC; results not shown) gave identical results.

In order to determine the extent of variability between these three groups, the major peaks from specimens 3, 4 and 6 were isolated for structural analysis. Furthermore, we could confirm the similarity of glycosylation between specimens 1, 2, 3 and 5, by also analysing major components from these specimens. After structural analysis, it appeared that specimens 1, 2, 3 and 5 formed a group presenting homogeneous glycosylation, while specimens 4 and 6 both presented an original glycosylation, as suggested by the chromatographic analyses. All the O-glycans structures analysed are described in Table 1. Depending on their

Table 4 ^{1}H NMR chemical shifts of the oligosaccharide alditols M to P

		Oligosaccharide alditols, δ (p.p.m.)			
O-Glycan	Reporter group	M	N	O	P
GalNAc-ol I	H-1,1′	3.78	3.78	–	–
	H-2	4.24	4.228	–	–
	H-3	4.023	4.045	–	–
	H-4	3.576	3.625	–	–
	H-5	4.15	4.188	–	–
	H-6,6′	3.84/3.463	3.925/3.695	–	–
	NAc	2.042	2.046	–	–
Gal-ol	H-1,1′	–	–	3.73/3.76	3.75
	H-2	–	–	4.18	4.187
	H-3	–	–	3.98	3.989
	H-4	–	–	3.89	3.892
	H-5	–	–	3.76	3.760
	H-6,6′	–	–	3.67	3.66
Gal(β1-3) II	H-1	4.699	4.682	–	–
	H-2	3.817	3.800	–	–
	H-3	4.182	4.204	–	–
	H-4	4.009	4.004	–	–
Gal(β1-3) III	H-1	4.893	4.877	–	–
	H-2	3.686	3.799	–	–
	H-3	3.891	4.189	–	–
	H-4	3.919	4.007	–	–
Gal(β1-3) IV	H-1	–	4.992	–	–
	H-2	–	3.694	–	–
	H-3	–	3.883	–	–
	H-4	–	3.903	–	–
Fuc(α1-2) F(II)	H-1	5.403	5.359	5.10	5.105
or	H-2	3.76	3.807	3.87	3.803
Fuc(α1-2) Gal-ol	H-3	3.76	3.697	3.81	3.87
	H-4	3.82	3.825	3.82	3.815
	H-5	4.30	4.327	4.21	4.205
	H-6	1.235	1.237	1.20	1.198
Fuc(α1-2) F(III)	H-1	5.326	5.390	–	–
	H-2	3.796	3.771	–	–
	H-3	3.73	3.856	–	–
	H-4	3.81	3.795	–	–
	H-5	4.33	4.270	–	–
	H-6	1.201	1.195	–	–
Fuc(α1-2) F(IV)	H-1	–	5.348	–	–
	H-2	–	3.782	–	–
	H-3	–	3.697	–	–
	H-4	–	3.82	–	–
	H-5	–	4.382	–	–
	H-6	–	1.219	–	–
GlcNAc(β1-6) II′	H-1	–	4.552	–	–
	H-2	–	3.717	–	–
	H-3	–	3.533	–	–
	H-4	–	3.434	–	–
	H-5	–	3.450	–	–
	H-6,6′	–	3.952/3.742	–	–
	NAc	–	2.039	–	–
GalNAc(α1-3) II	H-1	–	–	5.170	5.185
	H-2	–	–	4.304	4.308
	H-3	–	–	4.041	4.053
	H-4	–	–	4.080	4.080
	H-5	–	–	4.207	4.070
	H-6,6′	–	–	3.69	3.75
	NAc	–	–	2.06	2.062
GlcNAc(α1-3) III	H-1	–	–	4.95	4.900
	H-2	–	–	3.93	4.113
	H-3	–	–	3.85	3.878
	H-4	–	–	3.55	3.629
	H-5	–	–	4.12	4.183
	H-6,6′	–	–	3.83/3.73	3.85
	NAc	–	–	2.09	2.071

Table 4 (contd.)

O-Glycan	Reporter group	Oligosaccharide alditols, δ (p.p.m.)			
		M	N	O	P
Fuc(α1-3) F(III)	H-1	–	–	–	5.046
	H-2	–	–	–	3.722
	H-3	–	–	–	3.846
	H-4	–	–	–	3.804
	H-5	–	–	–	4.351
	H-6	–	–	–	1.169
NeuAc(α2-6)	H-3ax	1.681	–	–	–
	H-3eq	2.721	–	–	–
	NAc	2.033	–	–	–

presence, these structures were differentially assigned to each group.

Structural analysis

^{1}H NMR parameters of compounds A to W are summarized in Tables 2, 3, 4 and 5. Compounds A, D, I, J, M, O and Q have been described in *X. laevis* previously [11]. Briefly, the core of the A, D and J compounds was Gal(β1-3)[GlcNAc(β1-6)]GalNAc-ol as shown by the H-2 and H-5 proton resonances of GalNAc-ol [27]. Moreover, the set of the GlcNAc H-2, H-3 and H-4 proton resonances was typical of a terminal non-reducing residue. For compound A, the ^{1}H NMR parameters of Fuc were typical of the α-(1 $\rightarrow$ 2) linkage to Gal. Thus, the structure of A was deduced to be Fuc(α1-2)Gal(β1-3)[GlcNAc(β1-6)]GalNAc-ol. For compound J, the pattern of Fuc III and Gal III H-2, H-3 and H-4 proton resonances indicated a terminal Fuc(α1-2)Gal sequence, as observed for compound A. Furthermore, the unusually downfield-shifted value of Gal III anomeric proton at 4.89 p.p.m. was established as indicative of the sequence Fuc(α1-2)Gal(β1-3)[Fuc(α1-2)]Gal [11]. The compound D was established as an extension of A with a terminal Gal residue III linked in β-(1 $\rightarrow$ 3) to a Gal II. From the ^{1}H NMR parameters relative to I, the compound exhibited the same unit Fuc(α1-2)Gal(β1-3)[Fuc(α1-2)]Gal ascribed for J. However, the chemical shift of H-6 and H-6′ of proton resonances, and the downfield shift of H-5 proton resonance from the GalNAc-ol residue showed the absence of C-6 substitution on this residue. It has to be noted that Fuc II and Fuc III H-1 assignments reported in [11] have been revised on the basis of rotating-frame Overhauser enhancement spectroscopy ('ROESY') experiments (results not shown). This established the structure of compound I as the pentasaccharide Fuc(α1-2)Gal(β1-3)[Fuc(α1-2)]Gal(β1-3)GalNAc-ol. The structure of C has been reported to be a carbohydrate chain of the anuran species *Rana temporaria* [28]. Oligosaccharide D is an extension of C with a β-GlcNAc residue at C-6 of GalNAc-ol, as shown by the additional GlcNAc II' H-1 proton resonance and the characteristic chemical shifts of the GalNAc-ol H-5 signal.

Compound M has already been characterized in the oviducal mucin of *Rana dalmatina* [29]. Compound N (Figure 2) was isolated from jelly mucin of specimens 1 and 2. From the two-step relayed COSY spectrum depicted in Figure 2, the presence of three α-Fuc, three β-Gal, one β-GlcNAc and one GalNAc-ol units was clearly established. The characteristic chemical shifts of GalNAc-ol H-6, H-6′, as well as the H-2, H-3 and H-4 resonances of β-GlcNAc, are significant of the terminal GlcNAc in β-(1 $\rightarrow$ 6) linkage to GalNAc-ol. Thus, Fuc and Gal units are necessarily part of the lower branch of the molecule. The three anomeric protons observed at 4.682, 4.877 and 4.992 p.p.m. were assigned to Gal II, Gal III and Gal IV respectively. The set of H-2, H-3 and H-4 resonances of Gal IV are characteristic of the terminal unit Fuc(α1-2)Gal. The Gal II and Gal III H-3 and H-4 resonances are indicative of a 2,3-di-substituted β-Gal unit, while the Gal II H-1 signal is not significantly affected by the presence of the additional β-Gal IV unit. Moreover, the observation of the nuclear Overhauser effect ('NOE') contacts H-1 Fuc (IV)/H-2 Gal IV (results not shown) allowed us to determine the exact assignments of the anomeric protons of the three fucose units. These observations led to the proposal of the structure of compound N as an extension of compound J with a terminal Fuc(α1-2)Gal unit.

Structures O and Q were also characterized through a combination of NMR and methylation analysis in *X. laevis* previously [11]. The newly isolated compound R presented the same ^{1}H NMR parameters as Q for the Gal II, GalNAc III and GlcNAc IV units, but also showed an additional β-GlcNAc residue. The H-6 and H-6′ signals of the GalNAc-ol residue resonated at 3.917 and 3.711 p.p.m. respectively, which defines the presence of core type II. Thus, compound R appeared to be an extension of Q with a β-GlcNAc residue. The H-6 and H-6′ proton resonances of GlcNAc II' are deshielded at 4.373 and 4.23 p.p.m. respectively, and the absence of an additional couplage with ^{31}P confirmed the presence of a sulphate group attached at O-6 of this monosaccharide unit.

Compound T (Figure 3) presented identical characteristics to R but contained an additional β-Gal unit (Gal III') linked in (1 $\rightarrow$ 4) to the GlcNAc II' residue as shown by the deshielding of the GlcNAc II' H-5 resonance in compound T at 3.786 p.p.m. Furthermore, the downfield-shifted resonances of Gal III' H-3 and H-4 were attributable to the C-3 substitution by a sulphate group [30].

Oligosaccharide-alditol W contained two α-Fuc, two α-GalNAc, two α-GlcNAc, two β-Gal, one O-6 sulphated β-GlcNAc and one GalNAc-ol residues, as could be inferred from the integration of the anomeric signals and the set of vicinal coupling constants. The H-1 resonances of Gal II and Gal III', both observed at 4.709 p.p.m., were characteristic of a β-Gal unit involved in the 'blood group' A determinant [31]. These observations showed the structure of compound W to be an extension of R with the sequence GlcNAc(α1-3)GalNAc(α1-3)[Fuc(α1-2)]Gal.

Structures containing the terminal α-(1 $\rightarrow$ 4)-linked Gal unit

Compounds F, G and L have been described previously [11]. They were characterized by the presence of the Gal unit linked in

Table 5 ^{1}H NMR chemical shifts of the oligosaccharide alditols Q to W

nd, not determined.

O-Glycan	Reporter group	Oligosaccharide alditols, δ (p.p.m.)						
		Q	R	S	T	U	V	W
GalNAc-ol I	H-1	nd	nd	3.79	3.77	3.79	3.792	3.79
	H-1′	nd	nd	3.79	3.77	3.79	3.792	3.79
	H-2	4.310	4.287	4.287	4.30	4.325	4.293	nd
	H-3	nd	4.070	4.073	nd	4.069	4.068	4.071
	H-4	3.601	3.616	3.616	3.621	3.615	3.608	3.610
	H-5	4.22	4.19	4.192	4.191	4.190	4.188	4.198
	H-6	nd	3.917	3.924	nd	3.93	3.927	3.904
	H-6′	nd	3.711	3.712	nd	3.70	3.698	3.690
	NAc	2.038	2.043	2.043	2.044	2.044	2.044	2.047
Gal(β1-3) II	H-1	4.707	4.712	4.715	4.716	4.714	4.713	4.709
	H-2	nd	3.911	3.913	3.918	3.916	3.917	3.911
	H-3	nd	4.020	4.023	4.028	4.021	4.022	4.018
	H-4	4.22	4.209	4.219	4.207	4.216	4.215	4.206
GalNAc(α1-3) III	H-1	5.234	5.226	5.244	5.226	4.244	5.245	5.225
	H-2	4.328	4.339	4.348	4.337	4.348	4.347	4.337
	H-3	nd	4.067	4.058	4.043	4.058	4.057	4.042
	H-4	4.061	4.062	4.074	4.062	4.072	4.095	4.063
	H-5	4.158	4.24	4.211	4.231	4.27	4.232	4.278
	H-6	nd	nd	3.63	3.73	3.74	3.633	3.71
	H-6′	nd	nd	3.63	3.73	3.74	3.633	3.71
	NAc	2.046	2.043	2.049	2.047	2.052	2.052	2.056
GlcNAc(α1-3) IV	H-1	4.941	4.941	4.886	4.941	4.889	4.888	4.940
	H-2	nd	3.921	4.114	3.926	4.113	4.113	3.927
	H-3	nd	3.863	3.889	3.913	3.890	3.888	3.863
	H-4	3.549	3.549	3.642	3.550	nd	3.632	3.549
	H-5	4.112	nd	nd	4.173	nd	4.223	4.15
	H-6	nd	4.152	nd	3.85	3.845	3.848	3.828
	H-6′	nd	4.154	nd	3.722	3.845	3.742	3.737
	NAc	2.087	2.087	2.070	2.089	2.070	2.071	2.087
GlcNAc(β1-6) II′	H-1	–	4.588	4.589	4.614	4.614	4.629	4.602
	H-2	–	3.737	3.738	3.788	3.793	3.951	3.778
	H-3	–	3.557	nd	nd	nd	4.044	nd
	H-4	–	4.230	nd	nd	nd	nd	nd
	H-5	–	3.677	3.676	3.786	nd	3.836	nd
	H-6	–	4.373	4.371	4.450	4.452	4.416	4.379
	H-6′	–	4.23	4.236	4.320	4.324	4.378	4.26
	NAc	–	2.053	2.054	2.051	2.052	2.044	2.041
Gal(β1-4) III′	H-1	–	–	–	4.648	4.647	4.647	4.709
	H-2	–	–	–	3.671	3.673	3.622	3.871
	H-3	–	–	–	4.345	4.346	4.330	3.970
	H-4	–	–	–	4.294	4.296	4.276	4.221
GalNAc(α1-3) IV′	H-1	–	–	–	–	–	–	5.225
	H-2	–	–	–	–	–	–	4.310
	H-3	–	–	–	–	–	–	4.058
	H-4	–	–	–	–	–	–	4.042
	H-5	–	–	–	–	–	–	4.233
	H-6	–	–	–	–	–	–	3.72
	H-6′	–	–	–	–	–	–	3.72
	NAc	–	–	–	–	–	–	2.035
GlcNAc(α1-3) V′	H-1	–	–	–	–	–	–	4.940
	H-2	–	–	–	–	–	–	3.927
	H-3	–	–	–	–	–	–	3.852
	H-4	–	–	–	–	–	–	3.549
	H-5	–	–	–	–	–	–	4.15
	H-6	–	–	–	–	–	–	3.828
	H-6′	–	–	–	–	–	–	3.737
	NAc	–	–	–	–	–	–	2.087
Fuc(α1-2) F(II)	H-1	5.387	5.385	5.387	5.385	5.385	5.384	5.380
	H-2	nd	3.80	3.81	3.799	3.802	3.802	3.802
	H-3	nd	3.80	3.81	nd	nd	3.8	nd
	H-4	nd	nd	3.81	nd	nd	3.8	nd
	H-5	4.328	nd	4.327	4.319	4.327	4.322	nd
	H-6	1.232	1.228	1.231	1.232	1.230	1.235	1.226

Table 5 (contd.)

O-Glycan	Reporter group	Oligosaccharide alditols, δ (p.p.m.)						
		Q	R	S	T	U	V	W
Fuc(α1-3) F(IV)	H-1	–	–	5.052	–	5.054	5.055	–
	H-2	–	–	3.723	–	nd	3.720	–
	H-3	–	–	nd	–	nd	3.853	–
	H-4	–	–	nd	–	nd	nd	–
	H-5	–	–	4.356	–	4.357	4.356	–
	H-6	–	–	1.171	–	1.170	1.171	–
Fuc(α1–3) F(II′)	H-1	–	–	–	–	–	5.121	–
	H-2	–	–	–	–	–	3.679	–
	H-3	–	–	–	–	–	3.916	–
	H-4	–	–	–	–	–	3.796	–
	H-5	–	–	–	–	–	4.801	–
	H-6	–	–	–	–	–	1.181	–

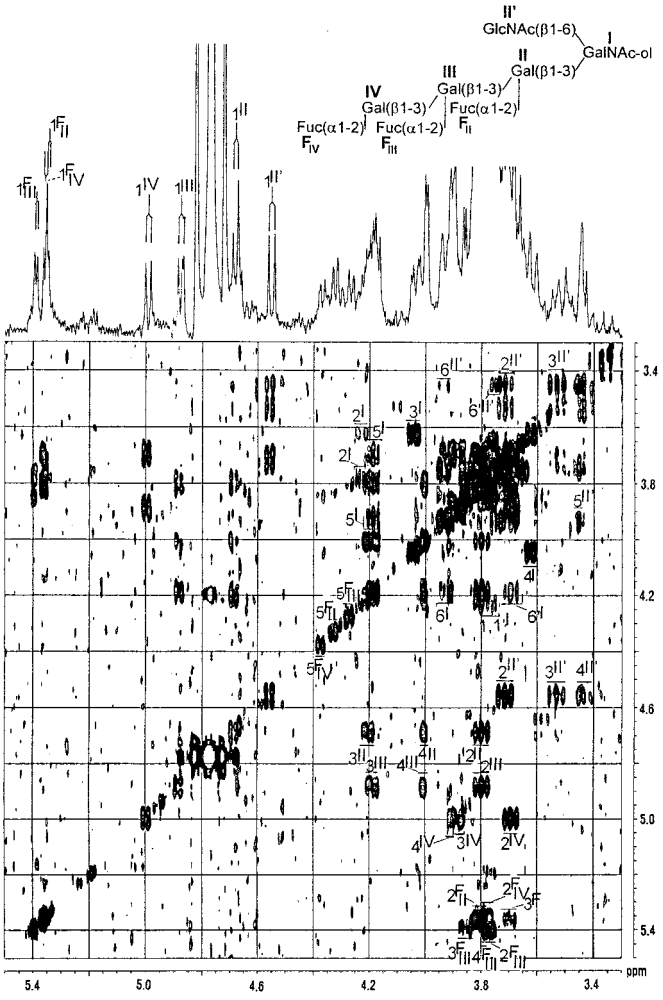


Figure 2 COSY spectrum of oligosaccharide alditol N

α-(1 → 4) to Gal II or Gal III. The compounds G (Figure 4) and L were extensions of A and J respectively, with an additional terminal Gal unit linked in α-(1 → 4). The Gal unit in an α-(1 → 4) linkage was clearly reflected by the anomeric proton observed at 4.932 or 4.937 p.p.m. for F and G respectively, and 4.98 p.p.m. for L, whereas the chemical shift of the H-5 signal varied between 4.32 and 4.38 p.p.m.

Compound H (Figure 4), which concomitantly presents both terminal Gal in an α-(1 → 4) linkage and terminal Fuc in an α-(1 → 3) linkage, are discussed below.

Structures containing the sequence Fuc(α1-3)GlcNAc(β1-6) or Fuc(α1-3)GlcNAc(α1-3)

Compounds B, E and K were extensions of compounds A, D and J with a Fuc linked in α-(1 → 3) to β-GlcNAc. Methylation analysis of compounds B, E and K confirmed this substitution through the presence of 4,6-di-*O*-Me-GlcNAc among the methyl ethers obtained from these compounds, while compounds A, D and J showed 3,4,6-tri-*O*-Me-GlcNAc. From the Tables 2 and 3, it can be noted that the chemical shifts of the Fuc H-1, H-5 and H-6 proton resonances are remarkably constant from one compound to another. Similarly, the structural-reporter-group signals of the constituting residues of the lower branch at GalNAc-ol match completely those of the same branch in A, D and J.

Four other compounds, namely P, S ,U and V, also contained an additional α-Fuc unit, characterized by its H-1 (δ5.05), H-5 (δ4.36) and H-6(δ1.17) proton resonances (Figure 3). A comparison of the COSY spectra of R and S on one hand, and S and T on the other hand, clearly indicated the sequence HSO_3(6)GlcNAc(β1-6) for R and S upper branches and HSO_3(3)Gal(β1-4)[HSO_3(6)]GlcNAc(β1-6) for T upper branch. The lower branches of compounds S and U were fucosylated at O-3 of α-GlcNAc, as proved by the downfield shift of its H-2 ($\Delta\delta = +0.19$) and H-2 ($\Delta\delta = +0.03$) signals. Thus they appear as an extension of the R and T lower branch with a Fuc in α-(1 → 3) linkage.

Methylation analysis was performed on compounds T and U to confirm the position of the Fuc in the α-(1 → 3) linkage. A mild periodate oxidation of GalNAc-ol residue of T and U, prior to the methylation, released the di-sulphated Gal(β1-4)GlcNAc sequence from the lower branch. Thus, the lower branch could be effectively extracted in the chloroform phase after methylation, while the sulphated upper branch remained in the aqueous phase. GC–MS analysis of compound T lower branch showed one 4,6-di-*O*-Me-Gal, one 2,3,4-tri-*O*-Me-Fuc, one 4,6-di-*O*-Me-GalNAc and one 3,4,6-tri-*O*-Me-GlcNAc among the partially methylated and acetylated methyl glycosides. Compound U differed from T by the presence of two 2,3,4-tri-*O*-Me-Fuc and one 4,6-di-*O*-Me-GlcNAc instead of a 3,4,6-tri-*O*-Me-

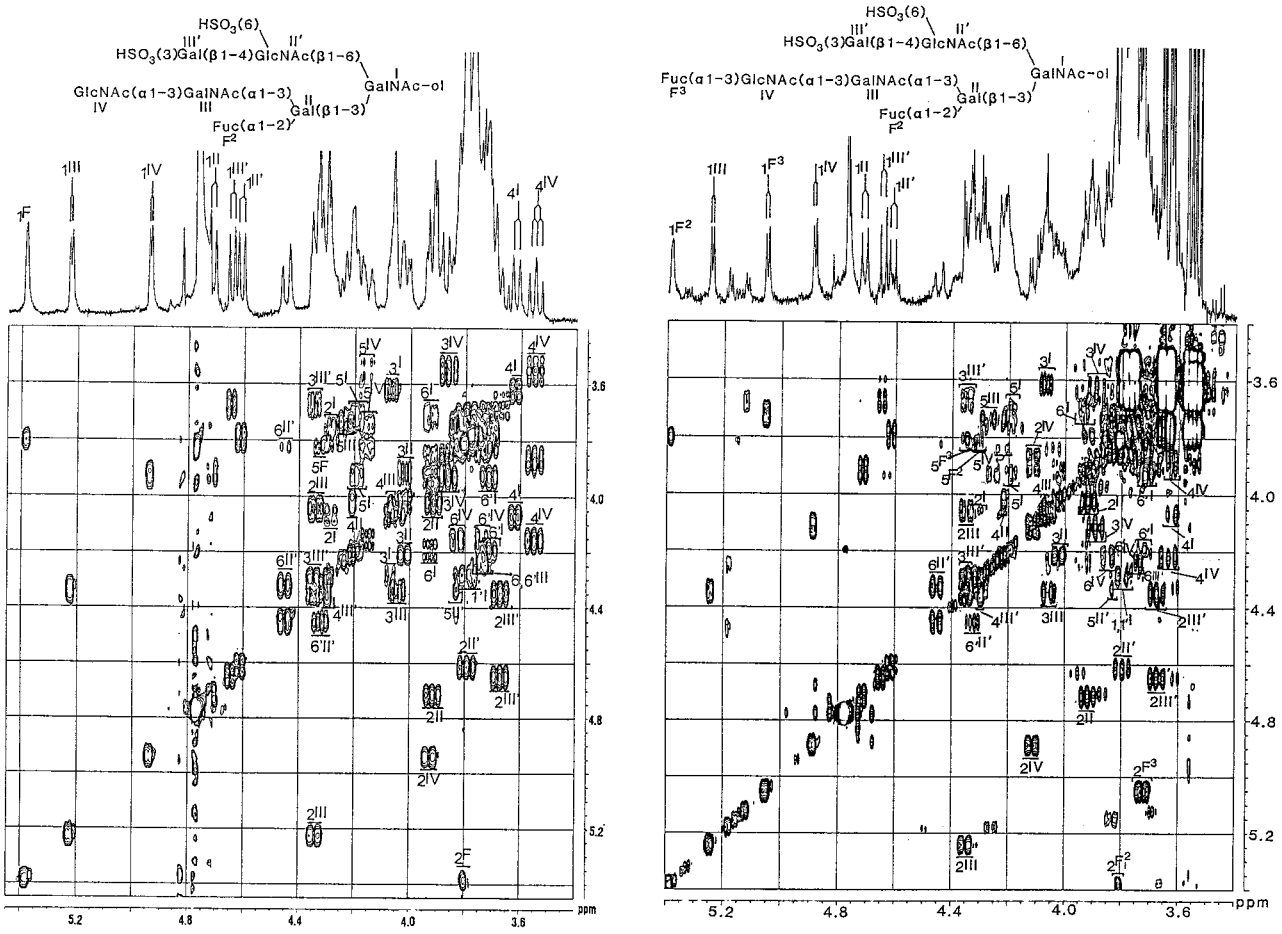


Figure 3 COSY spectra of oligosaccharide alditols T (left) and U (right)

GlcNAc. This confirmed the ^{1}H NMR parameters ascribed to Fuc in α-(1 → 3) linkage for compounds P, S, U and V.

Compound V contained two sulphate groups, attached to O-3 of β-Gal III' (δH-3 = 4.330; δH-4 = 4.276) and O-6 of β-GlcNAc II' (δH-6 = 4.416; δH-6′ = 4.378) respectively, as already described for compounds T and U. The presence of an α-Fuc unit O-3 linked to β-GlcNAc II' can be deduced from the Fuc H-1, H-5 and H-6 proton resonances, which are characteristic of the Lewis X determinant [27]. Since the NMR parameters relative to the lower branch perfectly match those observed for compounds S and U, the structure of compound V was fully established.

From these results, it appears that Gal in α-(1 → 4) linkage and Fuc in α-(1 → 3) linkage can be easily identified on the basis of one-dimensional ^{1}H NMR spectra. Particularly, the H-1 signals of α-Fuc O-linked to β-GlcNAc or α-GlcNAc are observed at 4.984–4.992 and 5.052–5.055 p.p.m. respectively. Similarly, the Gal in α-(1 → 4) linkage is characterized by its H-1 resonance observed at 4.932–4.936 or 4.98 p.p.m., according to the nature of the core, Gal(β1-3)GalNAc-ol (compounds F and G) or Gal(β1-3)Gal(β1-3)GalNAc-ol (compound L). Owing to these new structural reporter groups, the structure of compound H was established on the basis of the observation of signals at 4.936 (H-1 αGal) and 4.992 p.p.m. (H-1 αFuc). Compound H, which is a combination of B and G (Figure 4), is the one compound of the series exhibiting both Gal in α-(1 → 4) linkage and Fuc in α-(1 → 3) linkage.

Thus, as shown in Table 1, the three groups of *X. laevis* specimens differed by the terminal substitution of their egg-jelly O-glycans. Specimens 1, 2, 3 and 5 only presented Gal in α-(1 → 4) linkage, specimen 4 only presented Fuc in α-(1 → 3) linkage, while specimen 6 showed both substitutions.

Electrophoretic analysis

Extracted egg-jelly coats from the six specimens of *X.* laevis were analysed on a gradient 2.5–10 % polyacrylamide/SDS gel. As shown in Figure 5(a), PAS staining revealed three populations of very high-molecular-mass glycoproteins called Hm, Mm and Lm for high-, medium- and low-mobility glycoproteins respectively. It is noteworthy that all the O-glycans we have isolated from jelly, even the acidic ones, are susceptible to periodate oxidation and thus are stained by PAS. Lm populations showed typically two narrow bands, Mm populations a single broad band and Hm populations one to three thin bands. Mm and Hm populations present apparent molecular-mass variations between individuals. This polymorphic appearance is common to proteins with repetitive elements, such as mucin-type glycoproteins, where it is due to the expression of a variable number of tandem repeats [32] e.g. between 20 and > 100 in MUC 1 [33]. This phenomenon is

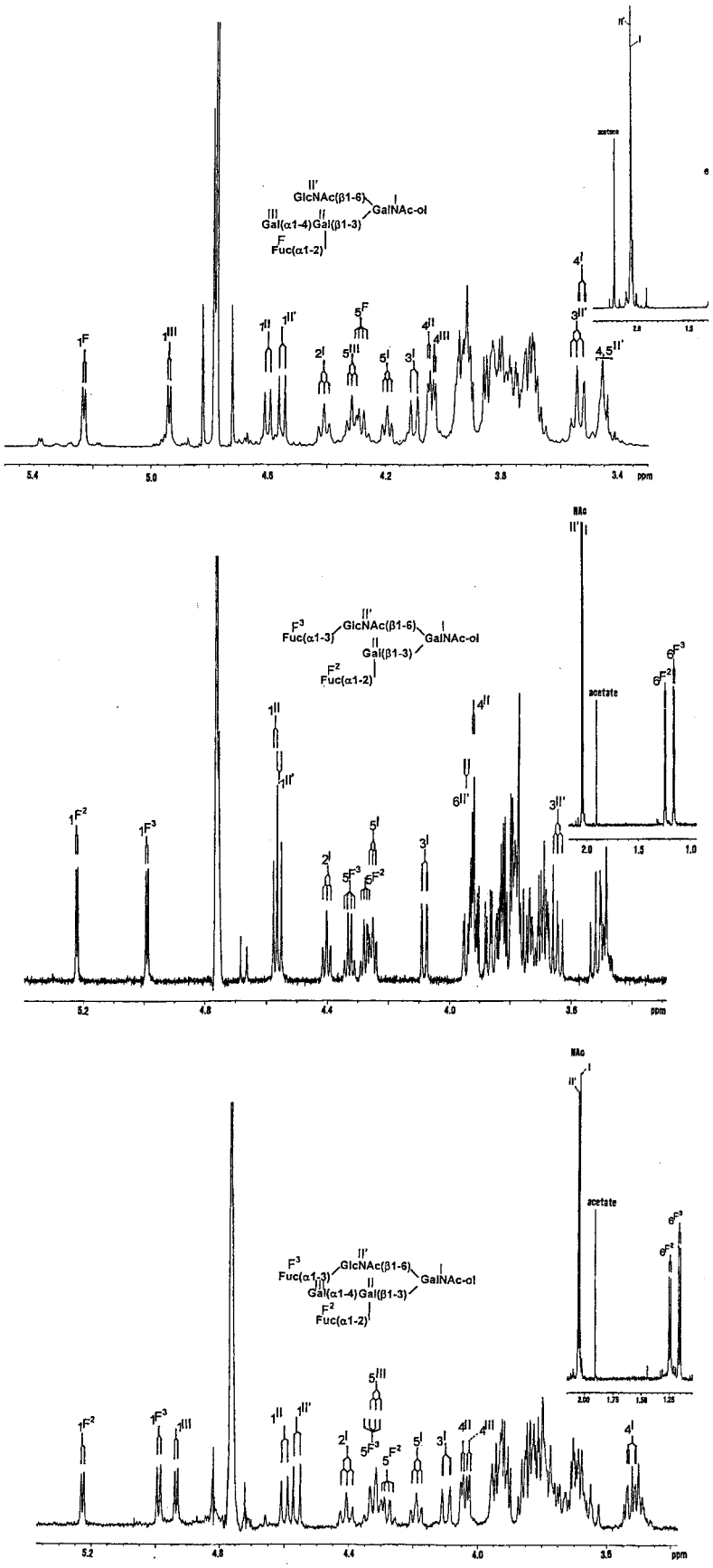


Figure 4 **^{1}H NMR spectra of oligosaccharide alditols G (top panel), B (middle panel) and H (bottom panel)**

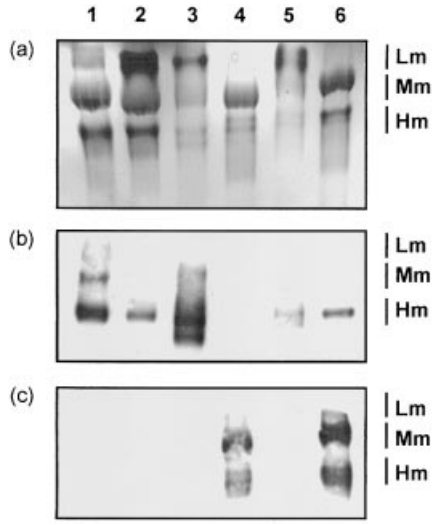


Figure 5 SDS/PAGE of egg jelly from six specimens of *X. laevis* (1 to 6)

A 2.5–10% polyacrylamide/SDS gradient gel was used. Gels were either (**a**) stained for carbohydrates with PAS or transferred on to nitrocellulose membranes. Membranes were then probed with either (**b**) BSI-B_4 lectin or (**c**) *T. purpureas* lectin. Lm, Mm and Hm: low, medium and high mobility respectively. In these experimental conditions, standard protein molecular-mass markers are excluded from the gel.

also typically responsible for the expression of polydisperse mRNAs in a single individual and synthesis of apomucins of different lengths. It has been observed for FIM B1 and FIM C1 in *X. laevis* [13,15] and for other mucins such as MUC 4 in humans [34]. The multiple bands observed in Lm and Hm populations may either result from the expression of such

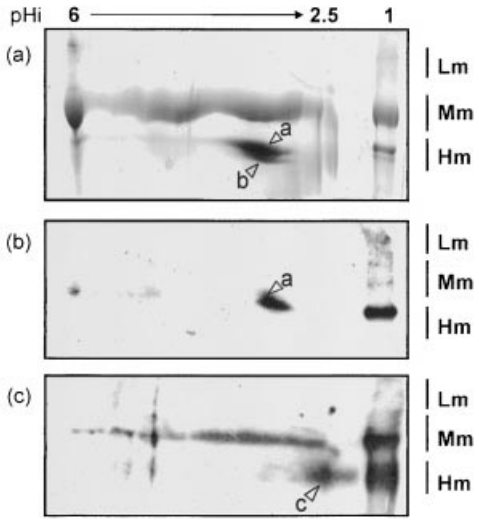


Figure 6 Two-dimensional electrophoresis of egg jelly from specimen 6 of *X. laevis*

Gels were either (**a**) stained for carbohydrates with PAS or blotted with (**b**) BSI-B_4 lectin or (**c**) *T. purpureas* lectin. Lane 1 shows the one-dimensional electrophoresis of specimen 6. Only the compound labelled 'a' in panels (**a**) and (**b**) from the Hm population reacted to BSI-B_4 lectin, while the compound labelled 'b' in panel (**a**) did not react to either lectin. A third compound from the Hm population, labelled 'c' in panel (**c**), reacted intensively to *T. purpureas* lectin.

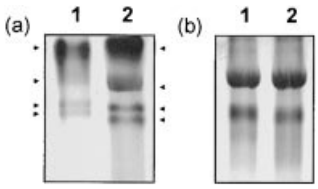


Figure 7 SDS/PAGE of egg jelly stained for carbohydrates with PAS

A 2.5–10% polyacrylamide/SDS gradient gel was used. (**a**) Jelly coat from specimen 2 was analysed before (lane 1) and after (lane 2) mild pronase proteolysis. For increased clarity the arrows indicate the exact positions of the bands for both samples. (**b**) Lane 1, total jelly coat from specimen 1; lane 2, high-density fraction ($\rho > 1.4$ g/ml.) recovered after CsBr ultracentrifugation of this sample.

polydisperse mRNAs or come from the synthesis of different components. The occurrence in specimen 4 and 5 of three bands in the Hm population is compatible with the first hypothesis considering the tetraploid status of *X. laevis* [35].

Two-dimensional electrophoresis of specimen 6 (Figure 6a) showed that the Hm population contained two very acidic components of similar pHi and slightly different apparent molecular-mass. The Mm population consisted of a mixture of molecules with different isoelectric points, at least two of which were acidic, while the L population appeared as not acidic.

In order to know whether the bands observed in SDS/PAGE resulted from molecular aggregation, despite the reducing conditions, samples were reduced and subsequently alkylated. We could not observe any difference in mobility on SDS/PAGE between these samples and their untreated equivalents (results not shown). This suggested that the high-molecular-mass components we observed were in their monomeric form.

After pronase digestion, the Mm and Hm populations of glycoproteins showed a slight reduction in their molecular mass (Figure 7a). However, we did not observe the appearance of new bands. These two observations are consistent with the presence of a protease-resistant, highly O-glycosylated core in these components. However, we did not observe any molecular-mass reduction for the Lm population in the pronase digested preparation. Considering the fact that this population barely penetrates the separating gel, it is likely that a slight reduction in molecular mass would not be visible under these experimental conditions.

After agarose electrophoresis (results not shown), PAS intensively stained one cathodic and one anodic broad band. The anodic PAS-reactive band showed the same mobility as purified bronchial mucins. Two anodic bands were stained by Toluidine Blue, one of which corresponded to the PAS stained anodic band. All these bands were entirely recovered in the fraction of upper density (superior to 1.4) after isopycnic ultracentrifugation. Furthermore, SDS/PAGE of this high-density fraction showed the same high-molecular-mass glycoprotein populations as the total egg-jelly coat (Figure 7b). The susceptibility of the anodic PAS-reactive band to Toluidine Blue showed that it contained acidic groups. Toluidine Blue stained bands were insensitive to any of the glycosaminoglycan degrading enzymes cited in the experimental section.

Thus, from these data, it appears that the PAS-reactive bands observed in agarose and polyacrylamide gels are typical mucin-type glycoproteins. Some of them exhibit acidic properties, which may be due to the presence of the sulphated O-glycans described previously, while others do not. This suggests that the mucin-type glycoproteins would support distinct O-glycans.

Also, the most anodic Toluidine Blue-reactive band does not appear as either a mucin-type glycoprotein, due to its lack of reactivity toward PAS staining, or a proteoglycan, as confirmed by its insensitivity to any glycosaminoglycan degrading enzymes.

Blot analysis

After SDS/PAGE and two-dimensional electrophoresis, gels were blotted and the membranes were probed with either BSI-B_4 or *T. purpureas* lectins, in order to localize α-galactose residues or Fuc in α-(1 → 3) linkage respectively, on egg-jelly glycoproteins.

As Figures 5(b) and 5(c) showed, we observed a perfect correlation between electrophoretic and structural, NMR based, analysis. Specimens 1, 2, 3, 5 and 6 were reactive to BSI-B_4 lectin, whilst specimens 4 and 6 were reactive to *T. purpureas* lectin. The *T. purpureas* lectin distinguished Fuc in α-(1 → 3) linkage residues from the ubiquitous Fuc in α-(1 → 2) linkage. Thus, the three phenotypes described previously are easily identified by this means. Systematic analysis of 13 other specimens of *X. laevis* permitted isolation of a specimen presenting the, not yet identified, [(α1-4)Gal (−); (α1-3)Fuc (−)] phenotype.

It appeared (Figures 6b and 6c) that the three populations of high-molecular-mass glycoproteins were not homogeneously substituted by Fuc in α-(1 → 3) linkage and Gal in α-(1 → 4) linkage. Indeed, except in specimen 1, where it also concerned the Mm population, α-(1 → 4) galactosylation was restricted to the Hm population of glycoproteins, while α-(1 → 3) fucosylation was more widely distributed in Mm and Hm populations. Figure 6(c) showed that a previously not described very acidic band from the Hm population-equivalent molecular-mass (label c) was most intensively substituted by fucose in α-(1 → 3) linkage. The Lm population did not support either substitution. Within the same population α galactosylation was not evenly distributed. Indeed, in specimen 6, only one band (label a), out of the two that the Hm population contained (Figures 5a and 5b), reacted with BSI-B_4 lectin.

Fertilization assays

Fertilization assays on eggs presenting [G(+), F(−)], [G(+), F(+)] and [G(−), F(−)] phenotypes repetitively showed scores of approx. 90%. So, no difference of fertilizability was observed between the three phenotypes tested. Fertilized eggs were kept for 6 h, and all of them showed normal subsequent development upon visual examination, irrespective of their phenotype.

DISCUSSION

In this study, we have fully sequenced 76 O-linked oligosaccharides isolated from the egg-jelly coat of six *X. laevis* specimens. We have described two families of O-glycans composed of 23 different structures, out of which 11 presented novel sequences. These O-glycans showed a remarkable polymorphism restricted to their terminal substitution. The combination of NMR-based and lectin studies led us to describe four phenotypes characterized by the differential expression of two glycosyltransferase activities, one α-(1 → 4) galactosyltransferase and one α-(1 → 3) fucosyltransferase activities, on the egg-jelly mucins. The Gal in α-(1 → 4) linkage substituted either the Gal II or the Gal III of one family of glycans, while the Fuc in α-(1 → 3) linkage substituted any α or β GlcNAc residue from both families. Out of 19 *X. laevis* specimens we have studied, 13 presented the [α-1,4Gal(+); α-1,3Fuc(−)] phenotype, four presented the [G(+); F(+)] phenotype, one the [G(−); F(+)] phenotype and one the [G(−); F(−)] phenotype. Each of these two activities may be the result of one or several glycosyltransferases. It should be noted that each substitution always occurs on the same acceptor substrate: Fuc(α1–2)Gal sequence for α-(1 → 4) galactosylation and GlcNAc residue for α-(1 → 3) fucosylation. Even so, transfer of Gal residue in α-(1 → 4) linkage on Gal II or Gal III may result from two distinct galactosyltransferases, while transfer of Fuc residue in α-(1 → 3) linkage on α-GlcNAc II or β-GlcNAc IV may also be due to distinct fucosyltransferases. Nevertheless, the fact that each substitution was always observed simultaneously in both positions in a specimen suggests that each one is the result of either a single glycosyltransferase, or more unlikely several glycosyltransferases which segregate 'en bloc' in each specimen.

To our knowledge, such a polymorphism has only be described for ABO, Hh and Lewis blood-group systems in humans. The resulting phenotypes observed in the ABO system and *X. laevis* jelly coat are very similar. Indeed, in both cases we observe four phenotypes due to different terminal glycosylations of complex oligosaccharide structures and which present the same pattern: [A(+); B(+)], [A(+); B(−)], [A(−); B(+)] and [A(−); B(−)] for ABO system, and [G(+); F(+)], [G(+); F(−)], [G(−); F(+)] and [G(−); F(−)] in *X. laevis*. However, it is most unlikely that the polymorphism observed in *X. laevis* results from the expression of allelic forms of a single gene, as observed in the ABO system [36,37]. Indeed, the polymorphism in *X. laevis* concerns both acceptor and donor substrates while it concerns only donor substrate in the ABO system. Furthermore, while all fucosyltransferases utilize GDP-associated fucose as the donor substrate [38], galactosyltransferases utilize UDP-associated substrate. Thus, considering the major differences observed between galactosyltransferase and fucosyltransferase activities, we postulate that the four phenotypes observed in *X. laevis* originate from the differential expression of at least two independent glycosyltransferases. Furthermore, it appeared that this polymorphism was tissue-specific. Western blot analysis (results not shown) showed that skin glycoproteins always presented α-linked galactoses but no fucose in α-(1 → 3) linkage, irrespective of the phenotype observed in egg jelly.

This work has brought new data about the exact nature of the high-molecular-mass fraction of *X. laevis* egg-jelly. It appears to be mainly composed of mucin-type glycoproteins. These components showed an important degree of inter- and perhaps intra-specific variability. The present study did not allow factors of variability such as synthesis of distinct apomucins, expression of polydisperse mRNAs and synthesis of glycoforms to be distinguished. However, considering the importance of sulphation in jelly O-glycans, it is most probable that the variations of pIs observed in Mm populations are due to variable degrees of sulphation in the constituting mucins. This was confirmed by the fact that we could separate two populations of mucins presenting different quantities of sulphate groups by CsCl centrifugation (results not shown). The presence of differently charged mucins was postulated by Yurewicz et al. [10] who observed that only the J_1 layer contained sulphate groups. Thus, components of Mm populations might consist of glycoforms differently substituted by sulphated O-glycans. Evidence of the synthesis of glycoforms of the same mucin within the same tissue was unambiguously demonstrated by the study of MUC 5B in bronchial secretions [39]. Blot analysis also showed that jelly mucins were differently glycosylated within the same specimen. In each jelly layer, synthesized by a distinct segment of oviduct [1], differential glycosylation may be the result of compartmentalization of glycosyltransferase activities in each segment or within a segment, or a strict regulation of glycosylation of each apomucin. The

oviduct of *X. laevis* appears as an appealing model for the study of O-glycosylation regulation because (i) important amounts of mucins are available, (ii) mucins which differ in their glycosylation are sequentially synthesized by distinct segments of oviduct, (iii) O-glycan structures synthesized by *X. laevis* oviduct are now well known, (iv) jelly mucins from *X. laevis* show a low extent of intra-specific variability of glycosylation, which is easily identified.

Only the diffusible components of egg jelly from *X. laevis* have a fertilization-promoting activity [7]. However, it was suggested that the ability of sperm to traverse the jelly was dependent on specific interactions with the carbohydrate moeities of jelly high-molecular-mass glycoconjugates [6]. Considering the important variation of glycosylation that we have observed in egg jelly from amphibians, it is conceivable that these sperm–jelly interactions would be very species specific. Thus, carbohydrate species-specificity may, at least partially, account for the inability of heterologous sperm to penetrate egg-jelly coat [4] and for the maintenance of the barrier preventing inter-species crossing. If so, divergence of glycan structures in egg jelly through evolution might be an important factor in speciation, by exerting an early control on inter-species fertilizability. The low extent of variation of glycosylation between the specimens presenting the [G(+); F(−)], [G(+); F(+)] and [G(−); F(−)] phenotypes did not seemed to influence fertilizability of the jellied egg. This suggests that Gal linked in α-(1 → 4) and Fuc linked in α-(1 → 3) in jelly are not involved in specific interactions with sperm. Thus, this intra-specific polymorphism showed by the jelly high-molecular-mass mucins does not seem to have an essential role in the control of egg fertilizability. It is not known to what extent O-glycan structures have to be modified in order to disturb sperm–jelly interactions and thus play a role in the selection of sperm.

Previous studies have shown that α-Gal-substituted glycans were ligands of the CGL [40] and were necessary for the formation of the fertilization envelope. The formation of this structure, as well as the cleavage of sperm receptors [41,42], is involved in the maintenance of monospermy in *X. laevis*. Despite this fact, eggs from specimens lacking terminal α-Gal substitution presented monospermic fertilization, as proved by their normal devolpment. Thus, either the fertilization layer was formed in the absence of the CGL putative ligand, or proteolytic cleavage of the sperm receptor alone is sufficient to maintain monospermy.

We believe that an in-depth knowledge of the egg-jelly matrix will help greatly in understanding the molecular mechanisms underlying the different interactions in which it is involved (sperm–jelly interactions, selection of sperm, formation of the fertilization layer). This will require studies of the structure of individual mucins and their glycosylation, as well as their exact location within the jelly.

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