Glycosylation of RNA polymerase II from wheat germ

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Abstract RNA polymerase II from wheat germ was analyzed for the presence of sugars. The two largest subunits and the 27 and 25 kDa subunits were found to be glycosylated by a variety of sugars. However, no *N*-acetylglucosamine was detected, which was found by Kelly et al. (J. Biol. Chem. (1993) 268, 10416–10424) in the largest subunit of RNA polymerase II from calf thymus. Thus it appears that the regulatory function of this sugar, postulated by Kelly et al., is performed in the wheat germ enzyme by other monosaccharides. Carbohydrate analysis of the two largest subunits of the calf thymus enzyme also revealed the presence, beside *N*-acetylglucosamine, of other sugars. Some similarities in the features of glycosylation of the two polymerases, isolated from very different organisms, suggest that the sugar moieties have an important role in the structure and/or function of these enzymes.

Key words: RNA polymerase II; Calf thymus; Wheat germ; Glycosylation

1. Introduction

RNA polymerase II is a high molecular weight enzyme with a complex quaternary structure, formed by the association of about a dozen different species of subunits, ranging from about 200 to less than 15 kDa. These features have so far hindered a detailed study of the mechanism of its action and of the functions of the various subunits. However, it is known that in vivo its enzymatic activity is precisely regulated by a variety of interacting proteins, and also by its own post-translational modifications, the best known of which is phosphorylation [1]. Recently a second type of modification has been described in the largest subunit of the enzyme from calf thymus, consisting in the glycosylation of multiple serine or threonine hydroxyl groups by single *N*-acetylglucosamine residues (GlcNAc) [2]. This modification appears to have an important regulatory role, since it inhibits the phosphorylation of the carboxyl-terminal domain of the polymerase subunit. It seemed interesting, therefore, to ascertain whether this type of glycosylation is a common feature of RNA polymerases II, and for this purpose we analyzed the enzyme from wheat germ. While we found no GlcNAc residues, we ascertained the presence of other sugars in significant amounts, not only in the largest subunit, but also in three other subunits.

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2. Materials and methods

2.1. Purification of RNA polymerase II and its subunits

RNA polymerase II was purified from wheat germ according to Jendrisak and Burgess [3] and from calf thymus by the method of Hodo and Blatti [4]. The isolated subunits were obtained by electroelution from 5 or 10% SDS gel electrophoresis with an Isco apparatus. To determine their concentration, aliquots of the isolated subunits were hydrolyzed in 6 N HCl at 110°C for 24 h and amino acid analyses were performed with a Pharmacia 4151 Alpha plus instrument.

2.2. Carbohydrate analysis

The sample was hydrolyzed at 100°C with 2 M trifluoroacetic acid (TFA) for 4 h to quantify neutral sugars, with 4 M HCl for 6 h for the amino sugars and with 0.05 N H_2SO_4 for 1 h at 80°C for sialic acid. Monosaccharide analysis was performed by anionic exchange chromatography with pulsed amperometric detection (HPAE-PAD, Dionex) [5–7].

2.3. Detection of RNA polymerase II with lectins

Wheat germ and calf thymus RNA polymerase II were electrophoresed through 5% or 12.5% SDS gels [8] and transferred to Immobilon membranes (Millipore) [9]. The blots were blocked in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 2% w/v bovine serum albumin for 1 h and then incubated with digoxigenin-labeled lectins (Boehringer) in the presence or absence of 1 M competing monosaccharide [10].

2.4. Labeling with galactosyltranferase

The subunits of RNA polymerase II were electroeluted from electrophoretic gels, and were reacted with 0.25–0.5 mU of bovine milk β-D-galactosyltranferase and 0.5 μCi of UDP-[^{3}H]galactose in GTase buffer (10 mM HEPES pH 7.4, 10 mM galactose, 5 mM $MnCl_2$, 0.5 M NaCl, 2% Triton X-100) in a final reaction volume of 45 μl on ice for 3 h [11]. To quantify incorporated galactose the proteins were adsorbed onto Immobilon membranes (Millipore). Free UDP-[^{3}H]galactose was washed off the membrane and radiolabeled galactose was estimated by scintillation counting of the entire membrane. Chicken egg ovalbumin (Sigma), chicken egg ovotransferrin (Sigma) and bovine serum albumin (Boehringer) were used as controls.

3. Results

3.1. Analysis by lectin recognition

The RNA polymerase II purified from wheat germ was analyzed by SDS-gel electrophoresis and its subunit composition was found to correspond to that described previously by Jendrisak and Burgess [12] (Fig. 1, lane 1). The largest subunit is known to be present in various subforms, with apparent molecular masses ranging from 205 to 180 kDa. With regard to the analysis of glycosylation they will be treated as a single form, and will be called the 200 kDa subunits. The subunits separated on gels were transferred on a membrane and tested with a variety of lectins. A recognition by at least one lectin was given by the two large subunits (i.e. 200 and 140 kDa) and also by those of 27 and 25 kDa. The lectin from *Galan-*

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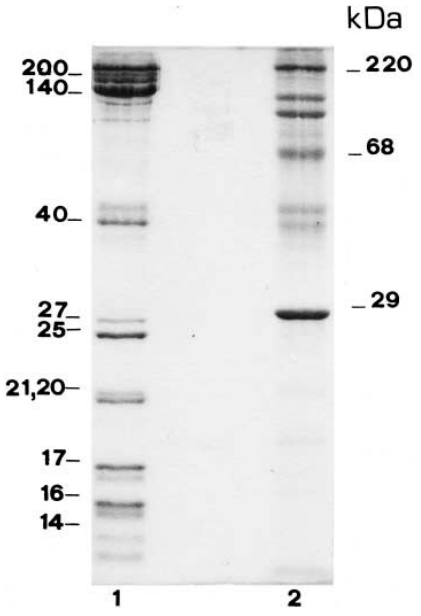

Fig. 1. SDS-polyacrylamide gel electrophoresis of RNA polymerase II from wheat germ (lane 1). Lane 2: molecular weight markers.

thus nivalis (GNA), which is specific for mannose residues, and particularly for high mannose structures, recognized all the above mentioned subunits. The other agglutinins tested, *Aleuria aurantia* agglutinin (AAA), *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA), reacted with some of the same subunits, as shown in Fig. 2. It is important to note that wheat germ agglutinin (WGA), which preferentially binds a terminal chitobiose or GlcNAc residues, did not recognize any subunit from the same enzyme.

3.2. Analysis of carbohydrate composition

The subunits recognized by lectins and shown in Fig. 2 were purified by SDS-gel electrophoresis followed by electroelution and their monosaccharide composition was determined after hydrolysis. As shown in Table 1, mannose (Man) residues were found in all cases, in agreement with the recognition by GNA. Sialic acid (Neu) and fucose (Fuc) were found in the subunits which were recognized by the corresponding lectins, i.e. MAA/SNA and AAA, respectively. Once again it should be noted that no GlcNAc was found in the subunits from wheat germ enzyme. To verify that its absence was not due to the hydrolysis conditions, 1 nmol of GlcNAc was added to the 200 kDa subunit and the mixture was hydrolyzed in 6 N HCl at 100°C for 4 h; in this case the GlcNAc was found (as GlcN) in a 90% yield. *N*-Acetylgalactosamine (as GalN) residues were present in the 200 kDa subunit and galactose (Gal) residues in the 200 and in the 27/25 kDa subunits.

Table 1
Monosaccharide composition of RNA polymerase II subunits determined by HPAE-PAD

Subunit	Monosaccharide	mol/mol
Wheat germ		
200 kDa	Man	19.8
	Gal	2.5
	GalN	1.3
	Fuc	0.6
140 kDa	Man	3.4
27/25 kDa	Man	2.5
	Gal	1.2
	Neu	1.3
Calf thymus		
200 kDa	Man	6.0
	GlcN	2.6
	Fuc	2.5
	Neu	7.0
140 kDa	Man	4.2
	GlcN	10.0
	GalN	2.0

3.3. GlcNAc analysis by galactosyltransferase

Galactosyltransferase catalyzes the transfer of galactose from UDP-galactose to accessible terminal GlcNAc residues

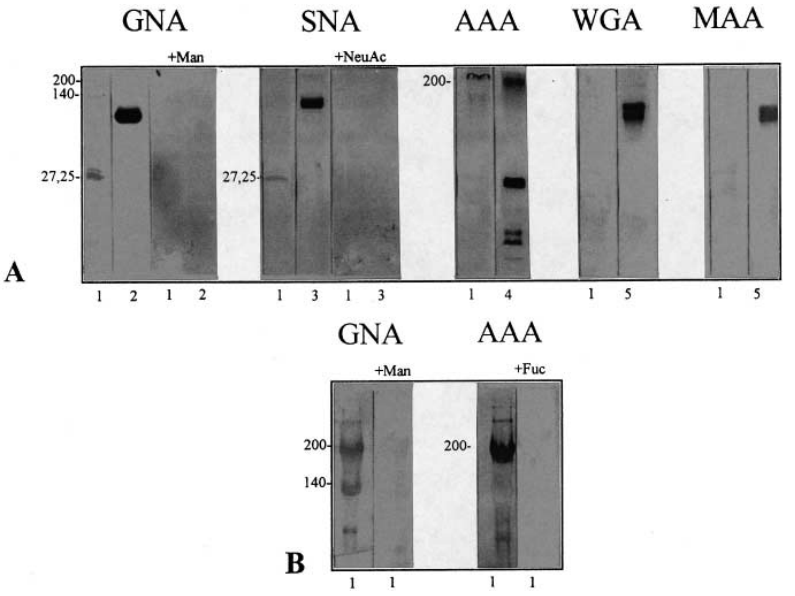

Fig. 2. Western blotting of the subunits of RNA polymerase II from wheat germ, tested with digoxygenin-labeled lectins in the presence or absence of competing sugars. A: SDS-PAGE at 12.5%. B: SDS-PAGE at 5%. Lane 1: RNA polymerase II; lane 2: carboxypeptidase Y; lane 3: transferrin; lane 4: bromolein; lane 5: fetuin.

bound to proteins. These residues can therefore be detected with high sensitivity by the use of UDP-[^{3}H]galactose. In order to check the performance of the method, ovalbumin, ovotransferrin and serum albumin were also subjected to the same procedure. As shown in Table 2, all these polypeptides gave the expected values of incorporated galactose. When this method was applied to the subunits of RNA polymerase from wheat germ which were recognized by lectins, it was found that none of them was affected by the reaction (Table 2).

3.4. Analysis of RNA polymerase II from calf thymus

The enzyme from calf thymus was purified according to Hodo and Blatti [4] and its subunits were analyzed for glycosylation. This enzyme was obtained in a smaller amount than that from wheat germ, and reproducible results for carbohydrates were obtained only for the two large subunits. The largest subunit, whose subforms IIo, IIa and IIb were not analyzed separately, will be referred to as the 200 kDa subunit.

GNA and WGA recognized both subunits, while MAA recognized only the largest, as shown in Fig. 3. The determination of monosaccharide composition after hydrolysis of the purified subunits indicated the presence of Man and GlcNAc residues in both subunits, plus fucose and sialic acid in the 200 kDa subunit (Table 1). Moreover, a small amount of GalNAc was found in the 140 kDa subunit.

The galactosyltransferase reaction showed that available GlcNAc residues were present only in the 200 kDa subunit, which incorporated 1.9 Gal residues per molecule, in good agreement with the data of Kelly et al. [2]. It is to be noted that all different forms (IIo, IIa and IIb) of the largest subunits of the latter enzyme were eluted together from an electrophoretic gel and then analyzed, without being separated, by the galactosyltransferase reaction.

4. Discussion

The results obtained demonstrate that RNA polymerase II from wheat germ is glycosylated in more than one of its subunits by a variety of monosaccharides. The same conclusion applies to the enzyme from calf thymus, even if in this case the analysis was limited to the two largest subunits.

Kelly et al. [2] have described the *O*-glycosylation by single residues of GlcNAc in the largest subunit (precisely in the subform IIa of this subunit) of the RNA polymerase II from calf thymus. Our data agree with this finding, since we

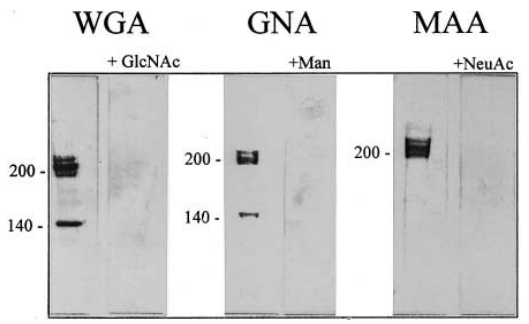

Fig. 3. Western blotting of the subunits of RNA polymerase II from calf thymus, tested with digoxygenin-labeled lectins in the presence or absence of competing sugars.

Table 2
[^{3}H]Galactose labeling

Protein	mol/mol[a]	mol/mol[b]
200 kDa w.g.	0.05	
140 kDa w.g.	0.09	
27/25 kDa w.g.	0.03	
200 kDa c.t.	1.9	2
140 kDa c.t.	0.6	
Ovalbumin	6.9	7
Ovotransferrin	7.6	8
Albumin	0.1	0

[a]Mol [^{3}H]Gal incorporated in our experiments.
[b]Expected values based on known compositions [1,13,14].
w.g. and c.t. indicate the subunits of wheat germ and calf thymus RNA polymerase II, respectively.

were able to detect the incorporation of galactose by the use of galactosyltransferase in the same subunit. We were also able to show that the latter is recognized by WGA, which is specific for GlcNAc. The only discrepancy with the data of Kelly et al. [2] is the fact that in our hands WGA also recognized the 140 kDa subunit of the same enzyme. Wether this is due to different reaction conditions with WGA in the Western blots, or to some other unknown reason, is not clear yet. However, the monosaccharide analysis which we performed showed that GlcNAc is also present in this subunit.

Kelly et al. [2] provided good evidence for a major regulatory role of the GlcNAc residues in the largest subunit. GlcNAc, which was shown to be present only in the carboxy-terminal domain in its unphosphorylated form, restricts the phosphorylation of serine residues, which in turn is an important event for the proper transcription process. This particular type of glycosylation has been found in a variety of proteins, often performing a regulatory function [15]. Considering the importance of this mechanism of biological regulation and its widespread occurrence, at least in animal cells, it might be expected to take place in all eukaryotes. However, glycosylation in plant cells, and particularly *O*-glycosylation, may differ from that occurring in animal cells. Thus, for example, Heese-Peck et al. [16] have recently described a nuclear pore glycoprotein from tobacco cells whose function appears to correspond to p62 protein of the vertebrate nuclear pore complex. While the latter is *O*-glycosylated with multiple, single GlcNAc residues, the plant protein is provided with *O*-linked oligosaccharides carrying terminal GlcNAc residues.

RNA polymerase II from wheat germ is totally devoid of GlcNAc, since we could not find it by any of three different methods: monosaccharide analysis after partial hydrolysis, WGA recognition and galactose incorporation by galactosyltransferase. While the negative result by the last method rules out the presence of terminal, exposed GlcNAc residues, the monosaccharide analysis demonstrated that this sugar is not present even in an internal, masked location. Therefore this plant RNA polymerase has neither the single GlcNAc residues of the mammalian enzyme nor the newly discovered oligosaccharide structure [16] with GlcNAc residues at the non-reducing end. Thus an important biological mechanism of regulation identified in animal cells might in plant cells be based on a different chemical modification of the proteins involved.

Galactose is often found as a serine-linked sugar in *O*-glycosylated plant proteins [17], and in fact Gal residues are present, together with other monosaccharides, in the 200 and 25/27 kDa subunits of wheat germ polymerase. Single

residues of this sugar have been found in other plants glycoproteins, and it would be tempting to suggest that single Gal residues in the 200 kDa subunit of this enzyme have the same regulatory function as the single GlcNAc residues found in the calf thymus enzyme.

The presence on both enzymes of various monosaccharides in more than one subunit suggests that the function of carbohydrates in RNA polymerase II is not limited to the regulatory role suggested by Kelly et al. [2]. Glycosylation might perhaps contribute to the folding and to the correct assembly of the nascent polypeptide chains. The importance of oligosaccharides for the process of folding has been demonstrated in several instances (reviewed in [18]). This could be an essential role in the case of RNA polymerase II, which has not only some high molecular weight subunits, but also a very complex quaternary structure.

It is also possible that carbohydrates facilitate the nuclear import of the massive polymerase molecule. In fact, Monsigny et al. [19] observed that, even in the absence of a nuclear localization signal, neoglycoproteins are able to enter the nucleus, in an ATP-dependent process. Otherwise, considering the need for RNA polymerases to act in vivo in association with other proteins, particularly with those constituting the multiprotein complex at the promoter site, the carbohydrates could represent the recognition sites for specific protein-protein interactions. However, no evidence is at present available leading to a decision among these, or even other [20], possibilities.

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References

[1] Sawadogo, M. and Sentenac, A. (1990) Annu. Rev. Biochem. 59, 711–754.
[2] Kelly, G.W., Dahmus, M.E. and Hart, G.W. (1993) J. Biol. Chem. 268, 10416–10424.
[3] Jendrisak, J.J. and Burgess, R.R. (1975) Biochemistry 14, 4639–4644.
[4] Hodo, H.G. and Blatti, S.P. (1977) Biochemistry 16, 2334–2343.
[5] Hardy, M.R. (1989) Methods Enzymol. 179, 76–85.
[6] Hardy, M.R., Towsend, R.R. and Lee, Y.C. (1988) Anal. Biochem. 170, 54–62.
[7] Ogawa, H., Ueno, M., Uchibori, H., Matsumoto, I. and Seno, N. (1990) Anal. Biochem. 190, 165–169.
[8] Laemmli, U.K. (1970) Nature 227, 680–685.
[9] Towbin, H., Stachelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[10] Haselbeck, A., Schickaneder, E., von der Eltz, H. and Hosel, W. (1990) Anal. Biochem. 191, 25–30.
[11] Torres, C.R. and Hart, G.W. (1984) J. Biol. Chem. 259, 3308–3317.
[12] Jendrisak, J.J. and Burgess, R.R. (1977) Biochemistry 16, 1959–1964.
[13] Dorland, L., Haverkamp, J., Vliegenthart, J.F.G., Spik, G., Fournet, B. and Montreuil, J. (1979) Eur. J. Biochem. 100, 569–574.
[14] Yamashita, K., Yoto, T. and Kobata, A. (1978) J. Biol. Chem. 253, 3862–3869.
[15] Haltiwanger, R.S., Busby, S., Grove, K., Li, S., Mason, D., Medina, L., Moloney, D., Philipsberg, G. and Scartozzi, R. (1997) Biochem. Biophys. Res. Commun. 231, 237–242.
[16] Heese-Peck, A., Cole, R.N., Borkhsenious, O.N., Hart, G.W. and Raikhel, N.V. (1995) Plant Cell 7, 1459–1471.
[17] Lamport, D.T.A., Katona, L. and Roering, S. (1973) Biochem. J. 133, 125–131.
[18] Fiedler, K. and Simons, K. (1994) Cell 81, 309–312.
[19] Duverger, E., Pellerin-Mendes, C., Mayer, R., Roche, A. and Monsigny, M. (1995) J. Cell Sci. 108, 1325–1332.
[20] Dwek, R.A. (1996) Chem. Rev. 96, 683–720.