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Purification and analysis of RNA polymerase II transcription factors by using wheat germ agglutinin affinity chromatography

(transcription factor Sp1/CCAAT-binding transcription factor/differential glycosylation/lectin binding/DNA affinity chromatography)

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ABSTRACT **We recently found that many RNA polymerase II transcription factors are modified with *N*-acetylglucosamine residues. These sugar moieties confer upon transcription factors an ability to bind the lectin wheat germ agglutinin. We have taken advantage of this interaction to devise a purification procedure for the "GC-box" binding transcription factor Sp1. Crude nuclear extracts are first subjected to wheat germ agglutinin affinity chromatography and then subjected to sequence-specific DNA affinity chromatography. The Sp1 protein purified by this procedure is at least 95% pure, and the overall recovery is >80%. In addition to yielding larger quantities of Sp1 than conventional schemes, the new purification procedure is also simpler and more rapid. We show that wheat germ agglutinin affinity chromatography can also be used to purify the glycosylated forms of the CCAAT-binding transcription factor. Thus, wheat germ agglutinin affinity chromatography may aid the purification of other transcription factors that bear *N*-acetylglucosamine residues. Furthermore, the ability to separate glycosylated forms of transcription factors from their unglycosylated counterparts by wheat germ agglutinin affinity chromatography should facilitate investigations into the role of *N*-acetylglucosamine residues in the functioning of transcription factor proteins.**

Transcriptional initiation by RNA polymerase II is modulated by the action of sequence-specific DNA binding proteins that recognize promoter and enhancer elements (1, 2). Since many of these sequence-specific transcription factors are present at low levels in the cell, it has generally been difficult to purify them in sufficient quantities for detailed analysis. During the past few years, however, a number of these transcription factors have been purified to near homogeneity through the use of sequence-specific DNA affinity chromatography (2, 3). One such transcription factor is the human Sp1 protein, which stimulates RNA synthesis from promoters that contain at least one correctly positioned Sp1 recognition site consisting of a G+C-rich decanucleotide sequence (the "GC-box"; ref. 4). Sp1 has been purified and shown to consist of two related polypeptides of ≈95 and 105 kDa (5). Subsequent isolation and sequencing of Sp1 cDNA clones, together with *in vitro* mutagenesis of the Sp1 coding region, has identified discrete regions important for Sp1 function. These consist of three "zinc finger" motifs, which mediate sequence-specific interaction with DNA (6), and multiple regions that confer an ability to activate transcription, including two glutamine-rich domains (7, 8).

Despite the relative wealth of information regarding Sp1 and several other transcription factors (1, 2), their mechanism of action is not yet understood in detail. It is hoped that the ability to express large quantities of eukaryotic transcription factors in bacteria and their subsequent analysis using *in vitro* reconstituted systems will lead to a better understanding of transcriptional activation. However, it has recently become clear that many transcription factors are posttranslationally modified and that at least some forms of modification are important in the regulation of transcription factor activity (9–12). Since eukaryotic proteins synthesized in *Escherichia coli* are unlikely to be appropriately modified, it will be necessary to purify transcription factors from homologous eukaryotic cells in order to study some aspects of transcription factor function. Although sequence-specific DNA affinity chromatography provides a means to obtain relatively pure preparations of DNA binding transcription factors from eukaryotic cells (3), generation of cell extracts before fractionation by such a procedure is often inefficient and laborious to perform. It would therefore be beneficial to have available alternative purification procedures for transcription factors, which are more efficient and rapid than those currently available.

Recently, a number of RNA polymerase II transcription factors were shown to possess multiple covalently attached *N*-acetylglucosamine (GlcNAc) moieties (9). For Sp1, these structures were shown to exist as O-linked GlcNAc monosaccharide residues. Interestingly, the *Triticum vulgaris* lectin, wheat germ agglutinin (WGA), binds tightly to Sp1 by means of these sugar residues and, in so doing, inhibits the transcriptional activation but not the DNA binding function of Sp1 (9). Here, we show that the use of WGA affinity chromatography can dramatically improve the speed and efficiency of transcription factor purification by circumventing many of the steps in standard purification schemes. Furthermore, the ability to separate glycosylated forms of transcription factors from their unglycosylated counterparts may be useful in analyzing the role of GlcNAc moieties in transcription factor function.

MATERIALS AND METHODS

Purification of Transcription Factors by Using Sequential WGA and DNA Affinity Chromatography. All procedures were performed at 4°C. Crude nuclear extracts were prepared from HeLa cells essentially as described by Dignam *et al.* (13), except that KCl rather than NaCl was used. Briefly, nuclei prepared from a 6-liter culture of HeLa cells [≈3×10^9 cells; 9 g of cells (wet weight)] were extracted with 30 ml of high salt buffer [50 mM Tris Cl, pH 7.5/0.42 M KCl/20% (vol/vol) glycerol/10% (wt/vol) sucrose/5 mM $MgCl_2$/0.1 mM EDTA/1 mM phenylmethylsulfonyl fluoride/1 mM sodium metabisulfite/2 mM dithiothreitol] and, after centrifugation at 80,000 × *g* for 1 hr, the supernatant fraction was collected and used as the source of transcription factor proteins.

For purification of Sp1, crude nuclear extract (derived from 9 g of HeLa cells; total protein ≈275 mg; 30 ml) was applied to WGA-agarose resin (Vector Laboratories; 2 ml

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Abbreviations: WGA, wheat germ agglutinin; CTF, CCAAT-binding transcription factor; NF-I, nuclear factor I.

preequilibrated in a Bio-Rad Econo-Column with high salt buffer) at a flow rate of ≈15 ml/hr. The resin was washed twice with 5 ml of high salt buffer and four times with 5 ml of Z′ buffer [25 mM Hepes-KOH, pH 7.6/12.5 mM $MgCl_2$/20% glycerol/0.1% (vol/vol) Nonidet P-40/10 μM $ZnSO_4$/1 mM dithiothreitol] containing 0.1 M KCl; then GlcNAc-bearing proteins were recovered by elution with 10 ml of Z′ buffer containing 0.1 M KCl and 0.3 M GlcNAc (Sigma). This eluate (10 ml; total protein ≈1.4 mg; ≈0.5% of the protein estimated to be Sp1) was mixed with sonicated calf thymus DNA (30 μg) and poly(dI)·poly(dC) (180 μg; Pharmacia), incubated for 15 min, and then subjected to sequence-specific DNA affinity chromatography with a resin bearing synthetic oligodeoxynucleotides containing the high-affinity Sp1 binding site 5′ GGGGCGGGGC 3′ (3). The quantity of nonspecific competitor described above is near the maximum that can be added without causing significant amounts (>5%) of Sp1 to flow through the DNA affinity resin. As little as one-fifth of this amount of competitor can be used without significantly reducing the purity of the final Sp1 preparation. Resins bearing succinylated WGA (Vector Laboratories), which is reported to have higher specificity for GlcNAc than underivatized WGA (14), can be used instead of WGA-agarose with essentially identical results.

WGA affinity purification of the glycosylated species of the CCAAT-binding transcription factor (CTF) was performed in an identical fashion to that described for Sp1 above, except that the crude nuclear extract was dialyzed against Z′ buffer containing 0.1 M KCl before application to the WGA-agarose resin and the resin was not washed with high salt buffer. In addition, only poly(dI)·poly(dC) (45 μg) was used as nonspecific competitor DNA for sequence-specific DNA affinity chromatography. The DNA affinity resin contained high-affinity CTF binding sites (15).

Detection of Proteins by Silver Staining and Western Blot Analysis. SDS/PAGE was carried out according to Laemmli (16) and silver staining of protein bands was as described by Morrissey (17). Proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose filters according to Towbin *et al.* (18). After incubation of nitrocellulose filters with anti-Sp1 or anti-CTF antibodies and treatment with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibodies (Pharmacia), as appropriate, antibody complexes were visualized by the method of Blake *et al.* (19). The anti-Sp1 antibody used was a mouse monoclonal antibody raised against a synthetic peptide corresponding to residues 520–538 of the Sp1 protein (S.P.J., unpublished data). CTF polypeptides were detected with a rabbit polyclonal antiserum (2897) raised against a synthetic peptide, which appears to be common to all the forms of CTF protein that are observed upon SDS/PAGE and silver-staining (N. Mermod, personal communication).

***In Vitro* Transcription and DNase I Protection Assays.** The activities of Sp1 and CTF preparations were tested by using DNase I protection assays and reconstituted *in vitro* transcription and primer extension assays (20).

RESULTS

Fractionation of Sp1 by WGA Affinity Chromatography. Standard purification schemes for Sp1 begin with the isolation of HeLa cell nuclei and extraction of Sp1 (and other transcription factors) from these nuclei with a buffer containing a fairly high concentration (≈0.42 M) of KCl (see *Materials and Methods*). In its simplest form, the purification proceeds with the precipitation of nuclear proteins by ammonium sulfate and then with fractionation of these proteins by Sephacryl S-300 gel filtration chromatography. These two steps are required in order to remove DNases prior to subsequent fractionation of the extract. Finally, an essentially pure Sp1 preparation is obtained by subjecting the Sephacryl S-300 eluate fractions that contain Sp1 to two or three successive rounds of sequence-specific DNA affinity chromatography (refs. 3 and 5; J. Kadonaga, personal communication).

If WGA affinity chromatography was to be useful in the purification of Sp1, we reasoned that most benefit would be gained by employing this procedure as early as possible in the purification scheme. We had previously shown that Sp1 immobilized on nitrocellulose filters could bind avidly to WGA, even in the presence of 0.1% (wt/vol) SDS, 0.5% (vol/vol) Triton X-100, and 0.5 M NaCl (ref. 9; S.P.J., unpublished data). These findings suggested that WGA would bind to the Sp1 in high salt crude nuclear extracts generated early in the standard Sp1 purification scheme. By Western blot analysis with anti-Sp1 antibodies (see *Materials and Methods*), we found that >95% of the Sp1 protein was retained upon passage of crude nuclear extracts through agarose resins bearing covalently attached WGA (Fig. 1, compare lanes 7 and 8 with lanes 9 and 10). By contrast, >99% of the bulk protein in these extracts flowed through the WGA-agarose resin under these conditions (Fig. 1, compare lanes 1 and 2 with lanes 3 and 4). Furthermore, bound Sp1 was quantitatively recovered from the WGA-agarose resin by elution with a buffer containing 0.3 M GlcNAc (Fig. 1, lanes 11–13), which is a competitive inhibitor of WGA (21). We have estimated that WGA affinity chromatography as employed above results in an ≈200-fold purification of Sp1 from crude nuclear extracts, with a yield of >90% (see legend to Fig. 1). It is estimated that Sp1 constitutes ≈0.5% of the protein in the eluate from the WGA affinity resin. Interestingly, the material that flows through the WGA affinity resin retains transcriptional activity for Sp1-unresponsive promoters and provides a rich source of general transcription factors depleted of Sp1, which can be used in reconstituted *in vitro* transcription reactions to study Sp1-dependent mRNA initiation (F. Pugh, personal communication).

Purification of Sp1 Using the Combination of WGA and DNA Affinity Chromatography. WGA affinity chromatography provided a powerful initial purification step for Sp1 (see above). In addition, we found that it could be used to rapidly concentrate Sp1 and transfer it from the high salt nuclear extract buffer into a buffer system suitable for subsequent sequence-specific DNA affinity chromatography. This was accomplished by passing crude nuclear extract through a WGA-agarose column, washing the column with high salt buffer and then with DNA affinity chromatography buffer (Z′ buffer; see *Materials and Methods*), and finally eluting Sp1 and other WGA-bound proteins with DNA affinity chromatography buffer containing 0.3 M GlcNAc. This eluate was found to be devoid of detectable DNase activity (S.P.J., unpublished data), indicating that passage of this material over a sequence-specific DNA affinity chromatography column would not destroy the DNA affinity resin. Moreover, since only glycosylated proteins were enriched by WGA affinity chromatography, the great majority of nuclear DNA binding proteins were removed by this step. Thus, WGA affinity chromatography provides an especially discriminating method for purifying specific DNA binding proteins and transcription factors that bear GlcNAc residues.

The eluate from the WGA-agarose column was mixed with nonspecific competitor DNA and was then subjected to sequence-specific DNA affinity chromatography using a resin bearing high-affinity Sp1 binding sites (ref. 3; see *Materials and Methods*). When a sample of the material generated by sequential WGA and DNA affinity chromatography was analyzed by SDS/PAGE and silver staining, ≈95% of the protein detected was found to reside in a doublet of bands of apparent molecular masses of 95 and 105 kDa (Fig. 2*A*, lanes 1 and 2; 3-fold more sample was applied to lane

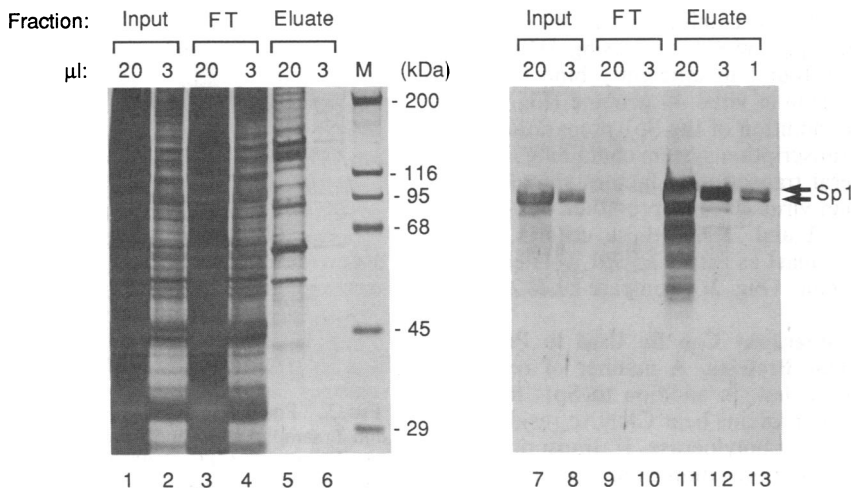

FIG. 1. Fractionation of Sp1 by WGA affinity chromatography. Duplicate samples of fractions generated by WGA affinity chromatography of a crude nuclear extract were analyzed on a SDS/8% polyacrylamide gel. The gel was then cut in half; one half (lanes 1–6) was stained with silver, whereas for the other half (lanes 7–13) proteins were transferred onto a nitrocellulose filter and Sp1 protein was detected by probing with an anti-Sp1 monoclonal antibody. Lanes 1 and 7 contain 20 μl (≈180 μg of total protein) of the crude nuclear extract that was applied to the WGA affinity resin (input fraction); lanes 2 and 8 contain 3 μl of the input fraction; lanes 3 and 9 contain 20 μl (≈180 μg of total protein) of the material that flowed through the WGA affinity resin (FT fraction); lanes 4 and 10 contain 3 μl of the FT fraction; lanes 5 and 11 contain 20 μl (≈2.8 μg of total protein) of the material eluted from the WGA affinity resin with 0.3 M GlcNAc (eluate fraction); lanes 6 and 12 contain 3 μl of the eluate fraction; and lane 13 contains 1 μl of the eluate fraction. M, protein standards (sizes in kDa). The locations of Sp1 polypeptides are indicated by arrows. From lanes 7–13, it is estimated that ≈95% of Sp1 in the input fraction is first bound to, then eluted from, the WGA-agarose resin. Total protein applied to the resin was ≈270 mg (180 μg in 20 μl; 30-ml total volume). Total protein eluted from the WGA resin with 0.3 M GlcNAc was ≈1.4 mg (2.8 μg in 20 μl; 10-ml total volume). Purification of Sp1 is therefore ≈200-fold (270 × 0.95/1.4).

1 than to lane 2). The identity of these species as Sp1 polypeptides was subsequently confirmed by Western blot analysis using anti-Sp1 antibodies. By comparing the level of Coomassie blue and silver staining of the Sp1 purified by sequential WGA and DNA affinity chromatography with that of protein standards, we have estimated the yield of Sp1 to be ≈40 μg from 9 g of HeLa cells (≈3 × 10^9 cells). Western blot analysis of various fractions generated throughout the Sp1 purification procedure indicated that the overall recovery of Sp1 was >80%.

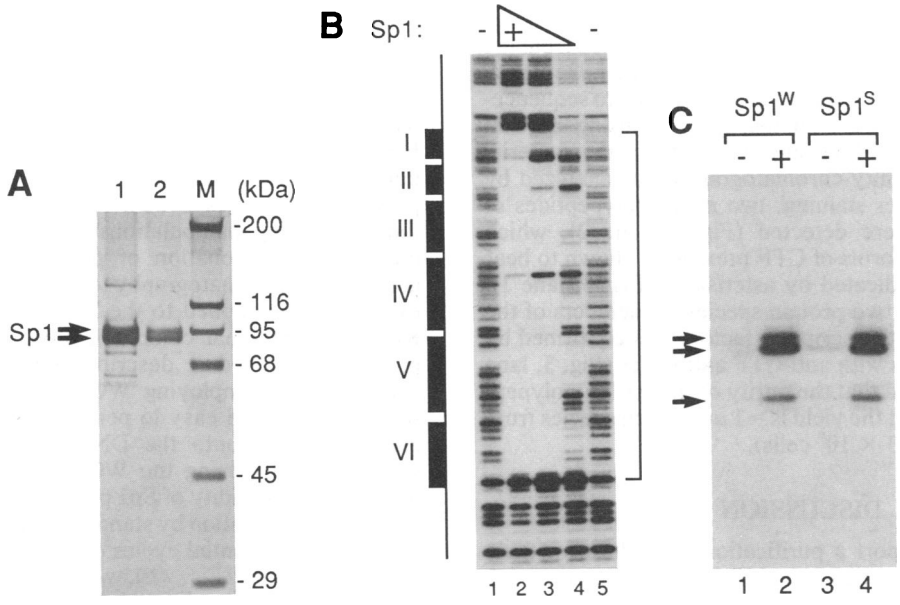

FIG. 2. Purification of Sp1 by the combination of WGA and DNA affinity chromatography. (*A*) Purification of Sp1. A crude nuclear extract derived from HeLa cells was subjected to WGA affinity chromatography followed by sequence-specific DNA affinity chromatography. Samples of the final Sp1 preparation (total volume of ≈2.2 ml) were then analyzed by SDS/PAGE and silver staining. Lanes 1 and 2 contain 20 μl (≈400 ng of Sp1 protein) and 7 μl (≈140 ng of Sp1 protein), respectively, of the purified Sp1 preparation. The two Sp1 polypeptides are indicated by arrows. M, protein standards (sizes in kDa). (*B*) DNase I protection analysis of Sp1 prepared by the combination of WGA and DNA affinity chromatography. The DNA probe used contained six tandem GC-box Sp1 binding sites (the 21-base-pair repeat region of the simian virus 40 genome, from pSV07; ref. 20). The relative positions of GC boxes I–VI are indicated to the left. The bracket to the right shows the region of the probe protected from DNase I digestion by bound Sp1. Assays were performed in the absence of Sp1 (lanes 1 and 5), with 20 ng (1 μl) of Sp1 protein (lane 2), with ≈4 ng of Sp1 (lane 3), or with ≈1 ng of Sp1 (lane 4). (*C*) Stimulation of *in vitro* transcription from the simian virus 40 early promoter by Sp1. Sp1 preparations generated by WGA-based and standard purification schemes are denoted by $Sp1^W$ and $Sp1^S$, respectively. Transcription reactions contained no Sp1 (lanes 1 and 3), ≈10 ng of $Sp1^W$ (lane 2), or ≈10 ng of $Sp1^S$ (lane 4). Arrows indicate primer-extended products of *in vitro* synthesized RNA.

When the Sp1 purified by the combination of WGA and DNA affinity chromatography was tested using DNase I protection assays, it was found to efficiently bind to the GC-box elements in the simian virus 40 genome (Fig. 2*B*, lanes 2–4). Furthermore, addition of the Sp1 preparation to a reconstituted *in vitro* transcription system containing RNA polymerase II and general transcription factors stimulated transcription of the simian virus 40 early promoter ≈30-fold (Fig. 2*C*, compare lanes 1 and 2). By these criteria, the WGA-purified Sp1 was at least as active as Sp1 isolated by standard purification schemes (Fig. 2*C*, compare lanes 2 and 4).

WGA Affinity Chromatography Can Be Used to Purify Other Transcription Factor Proteins. A number of recent reports have demonstrated that, in addition to Sp1, many other nuclear and cytosolic proteins bear GlcNAc residues. These include several RNA polymerase II transcription factors and a group of nuclear pore complex proteins (9, 22). We therefore investigated whether WGA affinity chromatography might be of general applicability in the purification of these and other GlcNAc-bearing proteins.

Of the several glycosylated proteins that we are currently studying, we chose to focus on the CTF/nuclear factor I (NF-I) family of transcription and DNA replication factors (15, 23). This group of proteins is particularly interesting, since only two of the multiple CTF polypeptide species appear to be glycosylated (9). Preliminary experiments indicated that the glycosylated forms of CTF bound weakly to WGA, if at all, when high salt crude nuclear extracts were applied to WGA-agarose columns (S.P.J., unpublished data). However, if crude nuclear extracts were dialyzed against buffers containing 0.1 M KCl before application to the WGA-agarose resin, an estimated 20% of the material comprising the forms of CTF previously shown to be glycosylated was selectively retained. [This was determined by Western blot analysis of input, flow-through, and eluate fractions from a WGA-agarose column by using anti-CTF antibodies (S.P.J., unpublished data).] The material that eluted from the WGA-agarose resin with 0.3 M GlcNAc was mixed with nonspecific competitor DNA and then subjected to sequence-specific DNA affinity chromatography by using a resin bearing CTF/NF-I binding sites (15). When the material purified by DNA affinity chromatography was analyzed by SDS/PAGE and silver staining, two major polypeptides of ≈62 and 64 kDa were detected (Fig. 3, lane 2), which comigrated with the forms of CTF previously shown to bear GlcNAc residues (indicated by asterisks in Fig. 3, lane 1). The identity of these two protein species as members of the CTF/NF-I family of transcription factors was confirmed by Western blot analysis with anti-CTF antibodies (Fig. 3, lane 4). We have estimated that the purity of these CTF polypeptides is ≈70% and that the yield is ≈1 μg of each species from 9 g of HeLa cells (≈3 × 10^9 cells).

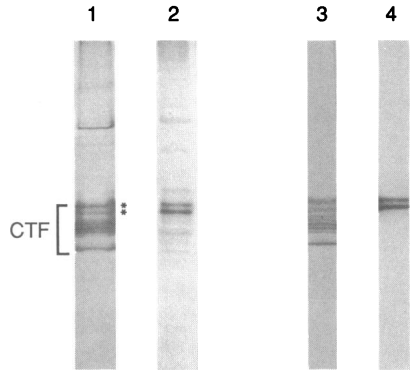

FIG. 3. Purification of glycosylated CTF polypeptides. For lanes 1 and 2, samples were analyzed by SDS/PAGE and silver staining. Lane 1 contains CTF (≈300 ng of total protein) purified by the standard CTF purification method (15). Lane 2 contains CTF (≈50 ng of total protein) purified by WGA affinity chromatography followed by sequence-specific DNA affinity chromatography. Lanes 3 and 4 are identical to lanes 1 and 2, respectively, except that instead of detection of proteins by silver staining, proteins were electrophoretically transferred to a nitrocellulose filter and the CTF polypeptides were detected with anti-CTF antibodies. The location of CTF polypeptides, which range from ≈52 to 66 kDa, are indicated by a bracket. Asterisks indicate the forms of CTF that were previously shown to bear GlcNAc moieties (9).

DISCUSSION

In this paper, we report a purification procedure for transcription factor Sp1, which is based on the finding that Sp1 contains covalently attached GlcNAc residues, whereas the vast majority of other nuclear proteins apparently do not (9, 22). This procedure can be divided into three stages (arrows in Fig. 4*A*). In stage 1, proteins are extracted from HeLa cell nuclei by a previously established method (13). In stage 2, the crude nuclear extract is subjected to WGA affinity chromatography. During this stage, Sp1 and other GlcNAc-bearing glycoproteins bind to the WGA affinity matrix, whereas >99% of the protein in the extract as a whole flows through the resin. Bound proteins are then eluted with GlcNAc, a competitive inhibitor of WGA. This step results in an ≈200-fold purification of Sp1, with a yield of >90%. In stage 3, the eluate from the WGA affinity resin is subjected to sequence-specific DNA affinity chromatography employing a resin bearing Sp1 binding sites. The resulting Sp1 preparation is >95% pure, and the overall yield for the purification procedure is >80%.

In addition to being much simpler and more rapid than previously described Sp1 purification procedures, the WGA-based purification method is also advantageous in that the yield of Sp1 is >4-fold higher than that from standard procedures. Furthermore, the Sp1 generated is generally purer and is at least as active at binding DNA and stimulating transcription *in vitro* as Sp1 purified by previous methods (Fig. 2 *B* and *C*). The improved performance of the WGA-based purification system is the result of several factors (see Fig. 4 for comparison of WGA-based and standard Sp1 purification procedures). First, previously described Sp1 purification schemes, even in their simplest form, require the time-consuming manipulations of ammonium sulfate precipitation and fractionation of proteins by Sephacryl S-300 gel-filtration chromatography to remove nucleases before the extract can be applied to the DNA affinity resin (ref. 5; J. Kadonaga, personal communication; see Fig. 4*B*). In the purification procedure described here, these steps are circumvented by employing WGA affinity chromatography, which is rapid and easy to perform. Second, the volume of material loaded onto the DNA affinity resin is reduced substantially by using the WGA-based procedure, further increasing the rapidity of Sp1 purification. Third, to generate a pure Sp1 preparation by standard Sp1 purification methods, at least two sequential cycles of DNA affinity chromatography are required (Fig. 4*B*), whereas with the WGA-based procedure, only one such cycle is necessary. Fourth, in standard Sp1 purification schemes, a relatively high level of nonspecific competitor DNA is required to prevent binding of nonspecific DNA binding proteins to the DNA affinity resin. This causes a large proportion (>30%) of Sp1 to flow through the DNA affinity resin, thereby substantially reducing yields. For the WGA-based purification procedure, however, smaller quantities of competitor can be used because the majority of DNA binding proteins are removed by the WGA affinity chromatography step. Due to this, <5% of the Sp1 flows through the DNA affinity column. Hence, WGA affinity chromatography is a much more discriminating

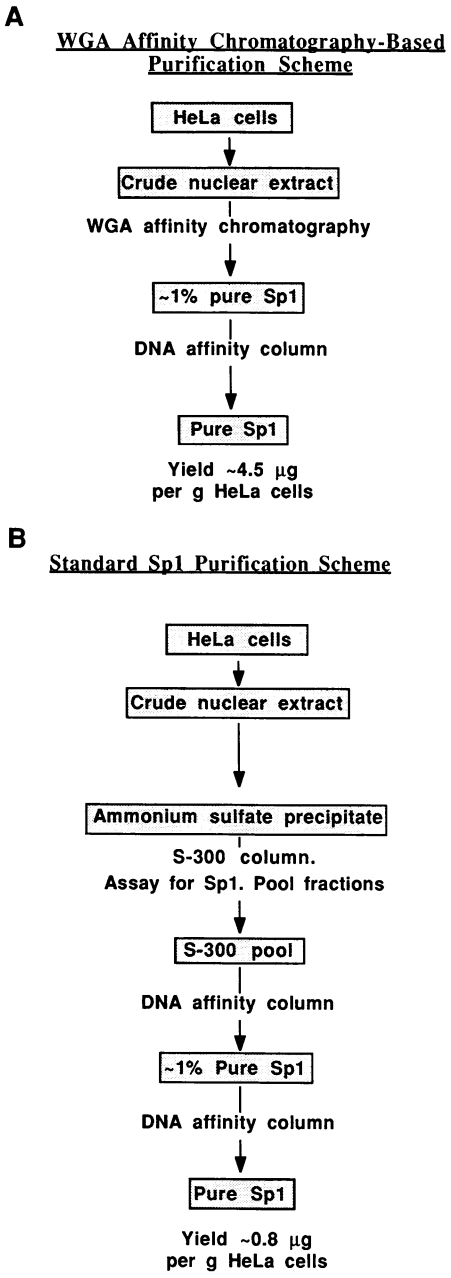

FIG. 4. Comparison of the WGA affinity chromatography based Sp1 purification scheme (*A*) with the standard Sp1 purification scheme (*B*).

method for initial enrichment of Sp1 and other glycosylated transcription factors than is nonspecific DNA affinity chromatography or chromatography on resins such as phosphocellulose or heparin-agarose, which also enrich for most other DNA binding proteins.

We have also shown that the combination of WGA and DNA affinity chromatography can be used to purify the glycosylated forms of the CTF/NF-I family of transcription and DNA replication factors to near homogeneity. Although the yield of CTF is not as high as that of Sp1, presumably due to CTF being glycosylated to a lower level than Sp1 (9), the amount of each CTF polypeptide purified is comparable to that obtained with standard CTF purification schemes (15). Furthermore, WGA affinity chromatography has recently been used to aid the purification of the liver cell-specific transcription factor HNF1 (S. Lichtsteiner and U. Schibler, personal communication). Although these findings suggest that WGA affinity chromatography might be useful in the purification of other proteins that bear GlcNAc residues, it should be noted that the presence of GlcNAc moieties does not necessarily confer strong binding to WGA. This is presumably due to the fact that avid binding by WGA requires multivalent interactions, which are dependent upon multiple GlcNAc residues being clustered together on the glycoprotein. Hence, WGA affinity chromatography might not be useful in the purification of all glycosylated transcription factors. A particularly noteworthy aspect of WGA affinity chromatography is its ability to separate the glycosylated forms of transcription factors from their unglycosylated counterparts. In this way, for example, it may be possible to determine whether certain biochemical or functional properties associated with transcription factors, such as the CTF family of proteins, are directly attributable to the presence or absence of GlcNAc residues. Thus, in addition to providing a convenient and rapid method for purification of certain transcription factors, WGA affinity chromatography may provide a biochemical tool to investigate the role of GlcNAc residues in the functioning of the eukaryotic transcriptional apparatus.

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