Magnetic DNA affinity purification of yeast transcription factor τ—a new purification principle for the ultrarapid isolation of near homogeneous factor

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ABSTRACT

We present a new method for rapid purification to near homogeneity of sequence specific DNA binding proteins based on magnetic separation. The method is described for the purification of the yeast transcription factor τ. DNA affinity Dynabeads (monodisperse superparamagnetic particles) specifically bind the protein in the presence of competitor DNA. By magnetic separation, wash and elution, highly enriched transcription factor preparations are obtained within minutes. In less than an hour with three cycles of adsorption, nearly homogeneous factor τ was obtained. The factor preparation contained mainly two polypeptides of 100 and 140 kDa and was fully active in transcription and DNA binding assays. This procedure should work for any high-affinity sequence-specific DNA binding protein with only minor modifications.

INTRODUCTION

When low abundance, unstable gene regulatory proteins are to be purified extensively, it is crucial with rapid and powerful purification techniques. Yeast transcription factor IIIC, given the name τ (1,2), is one of the most complex transcription factors characterized so far (2-6, reviewed in 7). This macromolecule interacts with a split binding site of variable spacing in the transcribed region on a variety of tRNA genes (2-6) through two domains on the protein, τ_A and τ_B (8). The factor is also required for 5S rRNA gene transcription (9, 10). Until now, purification to homogeneity and structural analysis of the factor have been impeded by low abundance and low yield using classical chromatographic procedures. This prompted us to develop a more efficient technique for the purification of τ factor using magnetic solid phase technology. The new method was found to be extremely rapid and powerful.

MATERIALS AND METHODS

Protein fractions

Transcription factor τ was purified from the *Saccharomyces cerevisiae* strain 20B-12 (*trp1, pep4-3*) by Heparin agarose, Gel filtration, DEAE Sephadex A25 chromatography and tDNA affinity column as previously described (1,2,6). As starting material for magnetic DNA affinity purification of factor τ, the Heparin agarose fraction was used while the Sephadex fraction was used to find optimal conditions for binding and elution. Purified RNA polymerase III was prepared as previously described (11).

Biotinylation of DNA.

A 200 bp EcoRI SalI fragment from plasmid pYtG-wt1 (12) containing the yeast $tRNA^{Glu}$ gene was purified using the FPLC/MonoQ system (Pharmacia). The $tRNA^{Glu}$ gene fragment was endlabeled at the SalI site using Bio21-dUTP (Clontech) and the Klenow fragment of DNA polymerase I (BRL). Unincorporated Bio21dUTP was removed using Sephadex G50 spin columns as described (13). The efficiency of biotinylation was visualized after agarose gel electrophoresis in the presence and absence of streptavidin in the samples.

Preparation of magnetic DNA affinity beads

25 mg prewashed Dynabeads™ M-280 Streptavidin (Dynal A/S, 0212 Oslo 2) were mixed with 20 µg of biotinylated $tRNA^{Glu}$ gene fragment in 150 µl TE buffer and placed in a roller (coulter mixer) at room temperature for 30 minutes. The efficiency of coupling was visualized by agarose gel electrophoresis of samples taken before and after coupling. Because of the high biotin binding capacity of Dynabeads™ M-280 Streptavidin (>200 pmol/mg), we usually got near 100% binding with the amounts of DNA given in the example above (6 pmol/mg). Significantly higher DNA densities on the beads can easily be obtained.

Optimalization of binding of yeast transcription factor τ to the DNA affinity Dynabeads.

Protein from a partially purified factor τ preparation (after the DEAE Sephadex step as described in 1) was mixed with nonspecific competitor DNA in buffer A (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 15 % Glycerol, and 0.05%

Nonidet P-40) containing the indicated amount of salt. A reference aliquot was usually taken at this stage to assay the amount of factor present before magnetic separation. To the mixture was added magnetic beads containing 0.2 μg $tRNA^{Glu}$ gene fragment per mg Dynabeads. When nonspecific adsorption was measured, Dynabeads™ M-280 Streptavidin with no DNA adsorbed was added to a separate mixture as a control. The incubation mixture was normally held at 25 °C for the indicated length of time before it was subjected to magnetic separation: A strong magnet (Neodynium-Iron-Boron Permanent Magnet (Dynal A/S, 0212 Oslo 2)) is placed against the wall of the tube, rapidly collecting the magnetic beads in a firm pellet. The "supernatant" can then be pipetted off while keeping the magnet in place. The presence of transcription factor in the supernatant was assayed by the electrophoretic DNA binding assay as described (14).

Optimalization of elution of yeast transcription factor τ from the DNA affinity Dynabeads.

The binding of transcription factor to the DNA affinity Dynabeads was performed as described above. After incubation and magnetic separation, the beads were washed by resuspention in buffer A containing 75 mM KCl, and factor activity was eluted with buffer A containing 1M KCl. Higher yield was obtained with highly purified factor preparations when 0.5 mg/ml bovine serum albumin was included in the washing and elution buffer.

Analytical purification of transcription factor τ by DNA affinity Dynabeads.

1.4 mg total protein from a partially purified factor τ preparation (after the Heparin agarose step (1,2)) was mixed with 200 μ g pUC19 plasmid in 2 ml buffer A containing 80 mM $(NH_4)_2SO_4$. 4 mg DNA affinity Dynabeads were added and the mixture incubated for 5 minutes at 25 °C. After dilution with 2 ml of buffer A, the magnetic separation was performed. The beads were then washed twice in 0.5 ml buffer A containing 75 mM NaCl and the factor eluted with 100 + 50 μ l buffer A containing 1 M NaCl (Eluate I, 18 μg total protein). The second cycle was initiated by mixing 120 μ l of eluate I with 10 μ g pUC19 plasmid and buffer A to a final volume of 800 μ l. The beads were then, after several washes in buffer A containing 2.0 M NaCl followed by a wash in buffer

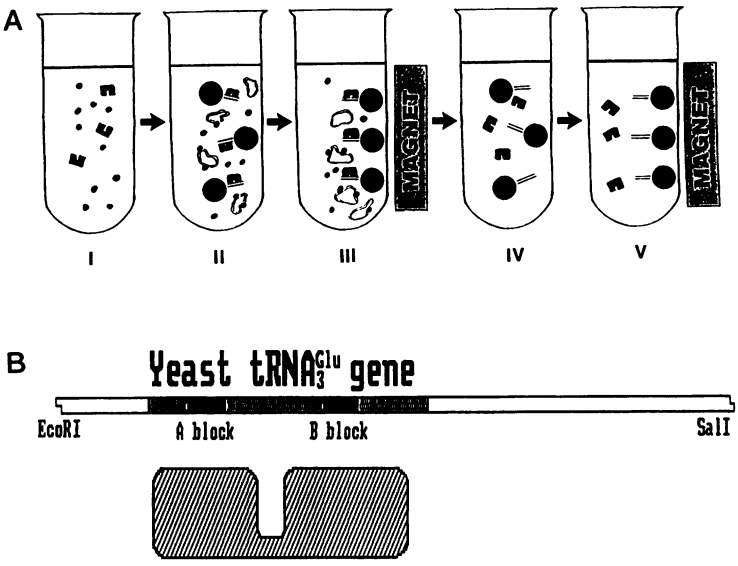

Fig.1 **Schematic representation of the principle of magnetic DNA affinity purification.** (**A**) **I**: The starting material, a partially purified protein fraction containing transcription factor τ. **II**: Adsorption of factor τ to the magnetic DNA affinity beads. **III**: Magnetic separation. **IV**: Elution with high-salt buffer. **V**: Magnetic separation. (▬): Sequence specific DNA binding protein (transcription factor τ), (●): Contaminating DNA binding proteins, (⊂⊃): Competitor DNA, (●═): Dyna-beads M-280 Streptavidin coated with biotinylated DNA containing the specific protein binding site. See text for further details.
(**B**): The $tRNA^{Glu}$ gene fragment coupled to Dynabeads™ M-280 Streptavidin after biotinylation of the SalI cleaved end. The shaded area indicates the region encoding the mature $tRNA^{Glu}$. The two conserved intragenic promoter elements, the A and B block, is shown in black. The cross-hatched figure below the gene illustrates the two-domain factor τ binding to the A and B block promoter elements.

A containing 150 mM NaCl, added back to this mixture and incubated for 5 min at 25 °C. After dilution with 800 µl of buffer A, the magnetic separation was performed. The beads were then washed twice in buffer A containing 75 mM NaCl and the factor eluted with 80 + 40 µl buffer A containing 1 M NaCl (Eluate II, less than 2 µg total protein). Finally, a third cycle was performed. 60 µl of eluate II was mixed with 5 µg pUC19 plasmid and buffer A to a final volume of 400 µl. The beads were then after high-salt wash and equilibration

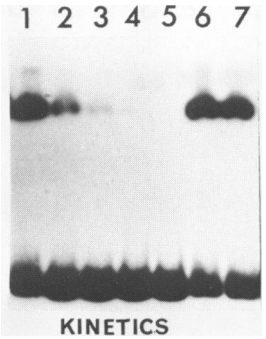

Fig.2 **Kinetics of factor τ removal by the DNA affinity Dynabeads.** 10 µg total protein from a partially purified factor τ preparation was mixed with nonspecific competitor DNA (10 µg pUC19 plasmid) in 500 µl buffer A containing 120 mM KCl, and 15 mM $(NH_4)_2SO_4$. 50 µl was taken out as a zero-time aliquot (lane 1). To 300 µl of the mixture was added 1 mg DNA affinity Dynabeads. To the rest was added 0.5 mg control beads (Dynabeads™ M-280 Streptavidin) with no DNA adsorbed. From the incubation mixture with the DNA affinity beads held at 25 °C, aliquots of 50 µl were taken after 1, 4, 10 and 20 minutes (lanes 2-5) and subjected to magnetic separation. From the control incubation mixture, aliquots were taken after 5 and 20 minutes (lanes 6,7). The presence of transcription factor in the supernatant was assayed by the electrophoretic DNA binding assay as described (14).

added back to the mixture and incubated for 5 min at 25 °C. 400 µl buffer A was added, magnetic separation performed, the beads washed and finally eluted with 60 µl as before (Eluate III, less than 1 µg total protein).

In vitro transcription.

The *in vitro* transcription system dependent on factor τ was reconstituted from partially purified TFIIIB, purified RNA polymerase III and the plasmid YtG-wt1 containing the yeast $tRNA^{Glu}$ gene as previously described (1). When the 5SrRNA gene was used as template, partially purified TFIIIA was included in addition. The source of the 5SrRNA gene was the plasmid p5S2 containing a 424 bp TaqI fragment subcloned in pUC19 containing the yeast 5SrRNA gene and 219 bp of upstream and 86 bp of downstream sequences.

DNaseI footprinting.

As probe for the DNaseI footprinting analysis was used a 200 bp fragment of the yeast $tRNA^{Glu}$ gene endlabeled with [α^{32}P]-dATP and the Klenow

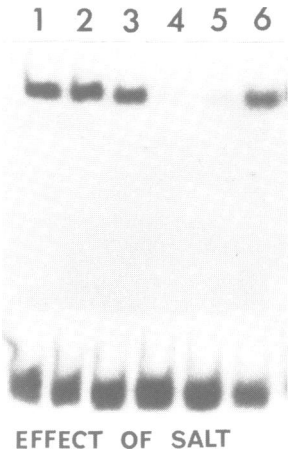

Fig.3 **The effect of salt concentration on factor τ removal.** Essentially as in Fig.2 using 100 µl incubations with 1.2 mg total protein, 1.2 mg pUC19 and 0.2 mg magnetic beads added and variabel salt concentrations. In addition to 15 mM $(NH_4)_2SO_4$, each incubation contained 20, 70, 120, 170, and 220 mM KCl (lanes 2-6). Incubation time was 10 min before magnetic separation. A control mixture with no magnetic beads added is shown in lane 1.

fragment of DNA polymerase I. The footprinting reactions were performed as previously described (2).

RESULTS

The principle and general strategy for the use of magnetic DNA affinity particles for the purification of sequence specific DNA binding proteins is diagrammed in Fig.1a. A specific DNA fragment, in our case a 200 bp fragment containing the yeast $tRNA^{Glu}$ gene (Fig.1b), is labeled in one end with a biotinylated deoxyribonucleotide. The biotin endlabeled fragment is bound to Dynabeads coated with streptavidin (Dynabeads™ M-280 Streptavidin) to give magnetic DNA affinity beads with high binding capacity. The beads are nonporous and the DNA is adsorbed to the surface, which is an advantage for the kinetics of protein binding. The biotin-streptavidin binding is extremely strong ($K_{ass}=10^{15}$ M^{-1} (15)) and resistant to high concentrations of salt and urea.

The sequence-specific DNA binding protein is bound to the beads by

incubation with a protein fraction (Fig.1a-I). A large excess of nonspecific DNA is added to reduce the binding of proteins with general DNA affinity to the beads (Fig.1a-II) . By placing a strong magnet against the wall of the tube, the magnetic beads with the specific protein attached is separated from contaminating proteins remaining in solution (Fig.1a-III) . This operation takes seconds. After a few washes with buffer at intermediate ionic strength, the specifically bound protein factor is eluted by resuspending the beads in a buffer of high ionic strength (Fig.1a-IV), incubating for a few minutes and removal of the beads by magnetic separation (Fig.1a-V). If the purity is not satisfactory after one adsorption, the method is rapid enough to be repeated in several cycles in less than an hour.

To purify yeast transcription factor τ by this method, magnetic streptavidin beads were loaded with the biotin-endlabeled $tRNA^{Glu}$ gene fragment to which the factor binds with an association constant in the order of 10^{10}-10^{11} M^{-1} (14). The kinetics of adsorption of factor τ to the affinity beads was monitored by the electrophoretic DNA binding assay (14) on the supernatant after magnetic removal of the beads. The affinity beads rapidly removed the factor from the mixture (Fig2), more than 50 % after 1 minute (lane 2) and more than 90% after 4 minutes (lane 3). No non-specific adsorption to the beads was detected after incubation of the factor with control streptavidin beads for five or twenty minutes (lanes 6 and 7). In general, the kinetics of binding will depend on the actual association constants and on the concentration of specific and nonspecific binding sites. Consequently, the kinetics will vary with the amount of magnetic affinity beads used, the DNA density on the bead surface and the concentration of competitor DNA. The minimal adsorption time necessary to achieve quantitative binding should be tested in each case.

It is critical to adjust the ionic strength to allow rapid exchange of factor between specific sites on the affinity beads and nonspecific sites on the competitor DNA in solution. A rather narrow range of ionic strengths was found to give rapid removal of factor τ (Fig.3). At an ionic strength of 50 mM (lane 2) and 100 mM (lane 3) most of the factor remained in the supernatant after 10 minutes in contact with the affinity beads. The kinetics of equilibration between

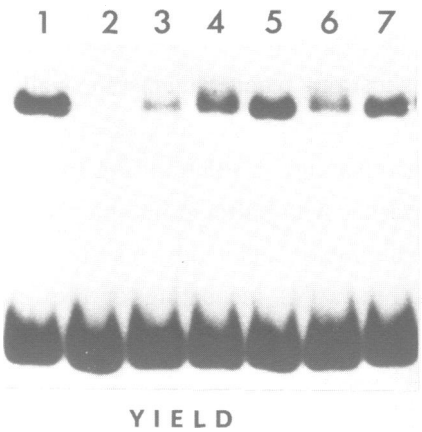

Fig.4 **Parameters affecting the yield of factor activity recovered from the DNA affinity beads.** 13 µg total protein was incubated in 250 µl buffer A containing 15 µg pUC19, 50 mM $(NH_4)_2SO_4$, 80 mM KCl, and 0.5 mg DNA affinity beads either at 25 °C (lanes 2,4,5) or on ice (lanes 3,6,7). Magnetic separation was performed after 10 min and residual factor τ activity tested (lanes 2,3). After washing the beads, factor activity was eluted with buffer A containing 1M KCl (lanes 4,6). In a parallel experiment 0.5 mg/ml bovine serum albumin was included in the washing and elution buffer (lanes 5,7). The initial DNA binding factor activity in an aliquot taken before addition of beads, is shown in lane 1.

specific and nonspecific binding sites is slow probably due to slow dissociation from the competitor DNA. At 150 mM (lane 4) and 200 mM (lane 5) most of the factor was adsorbed, suggesting optimal exchange kinetics. At 250 mM salt the factor does not bind well to the $tRNA^{Glu}$ gene (2) and most of the factor remains in the supernatant.

The yield of factor activity eluted from the beads was investigated at two temperatures with and without bovine serum albumin as a stabilizer (Fig.4). Preliminary experiments had shown that a salt-concentration above 0.4 M NaCl was sufficient to release factor activity, although higher yields were sometimes obtained above 0.8 M (not shown). The initial DNA binding factor activity in an aliquot taken before addition of beads, is shown in lane 1.When the adsorption was performed at 25°C quantitative factor removal was observed after 10 min (lane 2) as expected. When performed on ice, a small amount of factor was still

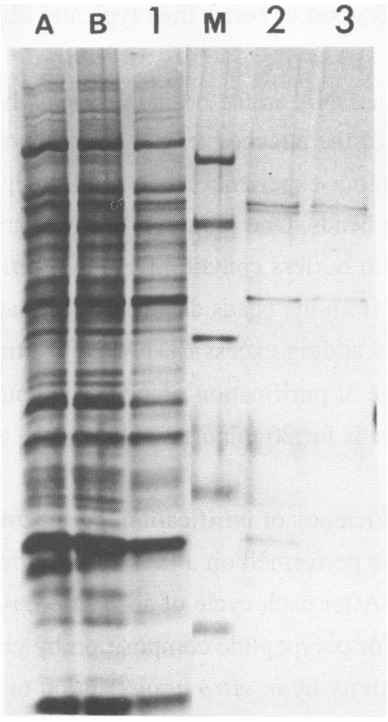

Fig.5 **Analytical purification of transcription factor τ by DNA affinity Dynabeads.** A partially purified factor τ preparation was bound to and eluted from the DNA affinity Dynabeads in three consecutive cycles as described in Materials and Methods. The efficiency of the purification and the polypeptide composition in each of the eluates were analysed by SDS-polyacrylamide gel electrophoresis (23) using the subunits of yeast RNA polymerase III as a size markers (lane M, visible bands from the top: 160, 128, 82, 53 and 40 kDa). Lane A and B: heparin-fraction before and after adsorption. Lanes 1-3: The three successive eluates (15 µl from each fraction).

present in the supernatant (lane 3) probably due to a slower adsorption kinetics. When the factor then was eluted from the beads by high-salt buffer, DNA binding activity was recovered. The yield of eluted factor was reasonable without stabilizer (lanes 4 and 6) and good with stabilizer (lanes 5 and 7). From Cerenkow counting of gelslices containing the retarded complexes, we estimated the yield of DNA binding activity recovered to be about 45% without stabilizer and 70% with stabilizer.The yield was not much affected by the temperature. In

our hands, this yield is as good or better than typically obtained using DNA affinity columns.

The stability of the DNA on the beads can easily be monitored by Klenow labeling of the free end of the attached fragment by ^{3}H or ^{32}P labeled deoxyribonucleotides. In our experience, the stability depends on the protein fraction mixed with the beads. One protein fraction at an early stage of purification prepared with buffers containing magnesium ions resulted in a significant loss of DNA from the beads during the incubation steps. This loss was only partially avoided by adding excess EDTA. By contrast, another protein fraction at a similar stage of purification prepared without divalent cations could be incubated with the beads for 20 minutes with no detectable loss of coupled DNA.

To analyse the efficiency of purification, the magnetic DNA affinity separation technique was performed on a partially purified factor τ heparin-fraction in three cycles. After each cycle of adsorption, wash and elution, aliquots were analysed for polypeptide composition by gel electrophoresis in the presence of SDS, for activity by *in vitro* transcription of both a tRNA and a 5S rRNA gene, and for DNA binding by DNase I footprinting. Fig.5 shows a silverstained SDS-polyacrylamide gel. When the heparin-fraction was analysed before and after factor removal no difference in band pattern was evident (lanes A and B), showing that the vast majority of the proteins remained in the supernatant. The degree of purification in the first eluate seemed modest (lane 1). The pattern of visible protein bands is very similar to that seen in the starting material. That a large amount of contaminating polypeptides nevertheless is removed is seen by comparing the relative amount of the total material loaded in each lane: 60-fold more is then loaded in lane 1 than in lane A. As judged from the silverstained gel, the second and third eluate (lane 2 and 3) retained mainly two polypeptides, 140 kDa and 100 kDa. These are exactly those previously found to specifically crosslink to the tRNA^{Glu} gene (6). In addition, only a minor band below 140 kDa is present in the third eluate. Comparable purity as in the second and third eluate has not previously been obtained with a native factor τ in solution. The fuzzy material around 60 kDa is a silver-stain artefact.

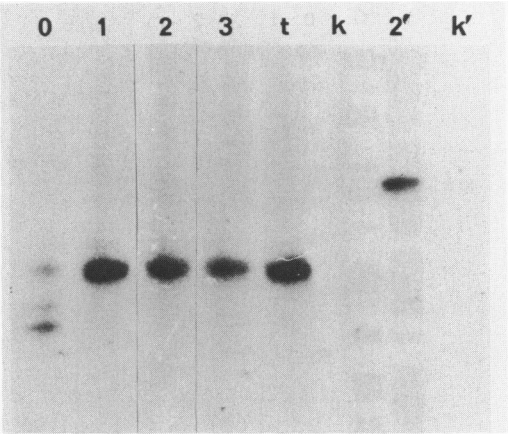

Fig.6 **Transcriptional activaty of factor τ purified by DNA affinity Dynabeads.**The factor τ activity purified as described in the legend to Fig.5 was assayed in a reconstituted transcription system (1). When a $tRNA^{Glu}$ gene template (lanes 0,1,2,3,t, and k) was transcribed the transcription assay was supplied with partially purified yeast TFIIIB and purified yeast RNA polymerase C. When a yeast 5S rRNA gene template was transcribed (lanes 2' and k'), partially purified yeast TFIIIA was also included. The fractions assayed was the initial heparin fraction (lane 0) containing tRNA processing activity, the three successive eluates (lanes 1,2,3 and 2'), a factor τ control (lane t) purified as previously described (2,4), and a control with no factor τ added (lanes k and k').

Thus, it appears that transcription factor τ could be composed of only two different subunits and have a $\alpha_x\beta_y$ structure.

Since the factor was isolated and assayed solely by virtue of its specific tDNA binding properties, it was important to show that an intact transcription factor had been isolated and not only its two DNA binding subunits (6).The near homogeneous preparation of factor τ was active in *in vitro* transcription both with a tRNA and a 5S rRNA gene as template (Fig.6). No specific transcripts were seen without factor τ added. This demonstrates that the ability to fully activate transcription resides in the polypeptides present in the final preparation. It also demonstrates that it is the same factor that activates the 5S rRNA gene and the tRNA gene and not two similar factors present in the partially purified preparations previously analysed for 5S rRNA gene transcription.

The factor purified by the magnetic DNA affinity technique also showed

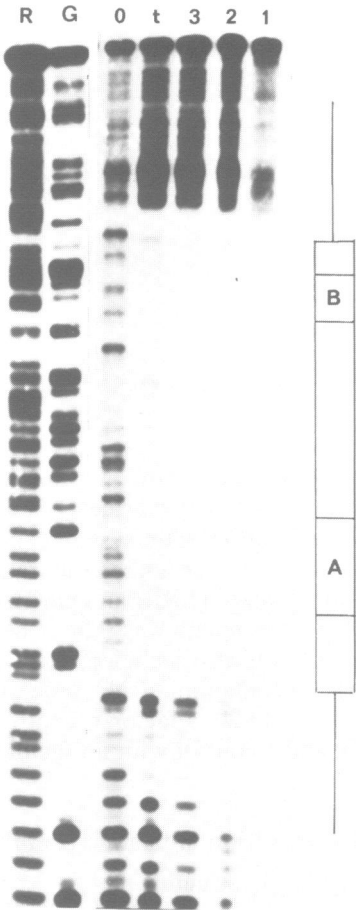

Fig.7 **DNaseI footprinting analysis of factor τ purified by DNA affinity Dynabeads.** The specific DNA binding activity of factor τ purified as described in the legend to Fig.5 was assayed by DNase I footprinting of the $tRNA^{Glu}$ gene as described (2). The fractions assayed was the three successive eluates (lanes 1,2,3), a factor τ control (lane t) purified as previously described (2,4), and a control with no protein fraction added (lane 0). Lanes R and G are the cleavage products of corresponding sequencing reactions (25) done on the same labeled DNA fragment.

the same DNaseI footprint as previously found on the $tRNA^{Glu}$ gene (Fig.7). The extention of the footprint into the upstream region seen with eluate 1, is probably a concentration phenomenon analogous to what have been seen with *Xenopus* TFIIIA at high concentrations (16).

Thus it appears that the two polypeptides of 140 and 100 kDa are sufficient to constitute a fully active τ factor, although we can not exclude the participation of the minor band just below 140 kDa. This could be a contaminating protein difficult to remove, a proteolytic degradation product of the 140 kDa polypeptide or a true subunit that stains weakly with silver.

Discussion

Very few transcription factors have been purified to homogeneity using classical column chromatographic methods. Examples are TFIIIA from *Xenopus* (17) and TFIIIB from HeLa cells (18). The introduction of DNA affinity chromatography using immobilized plasmids (2) or oligonucleotides (19) was a significant improvement that allowed the isolation of many sequence-specific DNA binding transcription factors in highly purified form, like the human factor Sp1 (20) and the cyclic AMP responsive element binding protein (21). The new method presented here is a further development of this affinity principle, where the slow column chromatography technique is substituted with much more rapid magnetic solid phase technology, a separation principle that has greatly simplified many immunological and cell-separation techniques (22,23). Furthermore, since the whole protein fraction is in contact with the affinity solid phase simultaneously, adsorption occurs with a kinetics approaching DNA binding in solution and thus contributes to the speed of the whole procedure. The binding occurs in a few minutes, the magnetic separation takes seconds, and the elution a few minutes. On scaling up, no proportional prolongation of time is necessary. The binding capacity is large. In the present work we coupled to the beads 6 pmol/mg of a small DNA fragment. Using biotinylated oligonucleotides up to 200 pmol/mg have been coupled. An alternative way of preparing enough DNA ready for coupling is by way of PCR amplification using one of the primers in a biotinylated form. The method was also found to be a powerful purification technique. In the case of transcription factor τ, higher purity was obtained in less than an hour than normally obtained with the same starting material after three days and three columns including a gene-affinity column. This procedure should work for any high-affinity sequence-specific DNA binding protein with only minor modifications.

The near homogeneous form of transcription factor τ obtained through this novel purification technique, seems to simplify our view of the structure of the transcription factor. The structure of factor τ was recently investigated by identifying the polypeptide chains specifically complexed to the $tRNA^{Glu}$ gene (6). Four polypeptides (145 kDa, 135 kDa, 100 kDa and 65 kDa) comigrated with the factor-gene complex in the electrophoretic DNA binding assay. Two of these (145 kDa and 100 kDa) were shown by UV-crosslinking to interact specifically with the $tRNA^{Glu}$ gene. Only antibodies raised against the same two polypeptides shifted the mobility of the factor-gene complex in the electrophoretic DNA binding assay. The 145 kDa polypeptide was found to belong to the τ_B domain that interacts specifically with the B block promoter element. From those data it was concluded that the 145 kDa and 100 kDa polypeptides were DNA binding subunits of the factor, but it was difficult to exclude the135 kDa and the 65 kDa polypeptides as part of the factor molecule. The present data strongly support a simple structure of the yeast factor τ with only the 140 and 100 kDa subunits being part of the factor protein, although the presence of a minor band slightly smaller than the 140 kDa polypeptide prevents a definite exclusion of a third component. The most purified fraction, where the two bands were the dominant polypeptides present, was fully active in transcription of both a tRNA gene and a 5S rRNA gene template, and no difference in the footprint pattern was observed when compared with less purified factor preparations. A simple two-polypeptide structure with one polypeptide interacting with each of the two promoter elements is a model that fits nicely with the two-domain structure of the factor derived from proteolysis experiments (8).

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