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A rapid method for the isolation of DNA-binding proteins from purified nuclei of tissues and cells in culture

Otto Hagenbüchle and Peter K.Wellauer*
Swiss Institute for Experimental Cancer Research, 1066 Epalinges, Switzerland

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ABSTRACT

We describe a rapid and general method for isolating DNA-binding proteins in high yield from purified nuclei of animal cells. The method has been tested for the isolation of a series of different DNA-binding activities including those of transcription factors PTF1 and SP1. The rationale consists of first preparing purified nuclei from tissue or cells in culture by centrifugation over sucrose cushions. A synthetic, biotinylated oligonucleotide bearing the binding site for the protein of interest is then added directly to nuclei resuspended in binding buffer. At the end of the binding reaction, nuclei are removed by centrifugation; and protein-DNA complexes present in the postnuclear supernatant are attached to streptavidin-agarose. Two rounds of DNA-affinity chromatography are carried out to yield highly purified preparations of DNA-binding proteins.

INTRODUCTION

Regulation of gene expression in eukaryotes and prokaryotes requires *trans*-acting protein factors that interact with *cis*-acting DNA sequences. The study of protein-DNA and protein-protein interactions is essential for the understanding of how active transcription complexes are established (for reviews see 1–5). A great number of DNA-binding proteins has been isolated during the past few years (for a recent compilation see 6). Several methods have been described by which DNA-binding proteins can be isolated. Common to all these methods is the preparation of a crude nuclear (7–10) or whole cell (11) lysate first. DNA-binding proteins are then purified from such protein extracts by chromatographic procedures. A major shortcoming of these protocols is that preparation of protein extracts is time consuming and not always reproducible. Furthermore, the purification of a particular DNA-binding protein from crude nuclear extracts may require the use of multiple chromatographic steps since the complexity of proteins in the starting material is high.

Here we report an alternative purification scheme that circumvents some of the problems encountered with traditional approaches. The method is fast and allows to reproducibly purify in high yields DNA-binding proteins of good quality.

MATERIALS AND METHODS

Preparation of crude nuclear extracts

Crude nuclear extracts (N.E.) from pancreatic cells in culture were prepared essentially as described by ref. 7 except that cell homogeneization and nuclear lysis buffers contained a cocktail of specific and non-specific protease inhibitors. These were added at the following final concentrations: 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 10 μM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), 8 μl/ml of Aprotinin (all from Sigma), 0.5 μg/ml each of pepstatin, antipain and leupeptin (all from Peptide Institute). Extracts (7–10 mg/ml of protein) were stored at −70°C.

Cell fractionation and purification of nuclei

For the preparation of nuclei from pancreatic AR42J cells in culture, cells were collected from culture media as described by ref. 20. All manipulations were carried out at 4°C from this stage on by using solutions, tubes and centrifuge rotors that were prechilled to 0°C. For homogenization, about 10^9 cells were resuspended in 20 ml of 0.3 M sucrose (Schwarz/Mann, RNase free) in buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM HEPES (pH 7.8), 14 mM mercaptoethanol and protease inhibitors described above). Deionized Nonidet P-40 (Sigma) was added to the cell suspension at a final concentration of 0.5% (may vary between 0.1 and 0.5% depending on the cell type) and cells were disrupted using an all-glass Dounce homogenizer (B-type pestle). When more than 95% of the cells were lysed as judged by light microscopy, the homogenate was layered on top of a 10 ml cushion of 0.9 M sucrose in buffer A and centrifuged for 10 min at 3.5 krpm and 4°C in a Sorvall HB-4 rotor. Glycerol (Merck, analytical grade) was added to the supernatant at a final concentration of 10% (v/v). The supernatant (cytoplasmic fraction) was stored at −70°C or used directly for the isolation of cytoplasmic poly A^+ RNA. The nuclear pellet was resuspended in 20 ml of 0.3 M sucrose in buffer A and 0.2% Nonidet P-40 by homogenization in a Dounce homogenizer (3 strokes, B-type pestle) and recentrifuged over 10 ml 0.9 M sucrose. The pellet containing purified nuclei was resuspended in 1 ml of buffer B (75 mM NaCl, 0.5 mM EDTA, 20 mM Tris·HCl (pH 7.9), 0.8 mM dithiothreitol (DTT), 0.1 mM PMSF and 50% glycerol) by repeated passages through

* To whom correspondence should be addressed

a micropipette tip. An aliquot of the sample was used for the counting of nuclei in a cell counting chamber in the microscope. The bulk of the sample was centrifuged for 3 min at 3.5 krpm and 4°C in the HB4 rotor and sedimented nuclei were resuspended at a concentration of 5×10^5 nuclei/μl in buffer B. This nuclear suspension was used directly for protein-DNA binding studies or was stored at −70°C prior to use. We have used nuclei that have been stored in buffer B for prolonged periods of time (several years) for binding studies without detecting a significant loss of the DNA-binding activity of transcription factor PTF1. The ability to store nuclei greatly facilitates the isolation of low abundance DNA-binding proteins from cells in culture since nuclei can be accumulated in batches over time. For the purification of nuclei from animal tissues a modification of the above protocol was used. Tissue in 0.3 M sucrose in buffer A was homogenized by 12 strokes in a motor driven teflon/glass homogenizer to yield a 10% (w/v) homogenate. The homogenate was filtered, first through 100 μm and then through 40 μm mesh gauze, and centrifuged over a 0.9 M sucrose cushion. Pelleted nuclei were homogenized in the presence of 0.2% Nonidet P-40 as described above and recentrifuged. The treatment of nuclei with the detergent is essential for the leakage of proteins out of nuclei.

Preparation of non-specific DNA competitor

DNA was extracted from *E.coli* cells according to ref. 21 with the following modifications: chloroform was used instead of chloroform/isoamylalcohol; after the first ethanol precipitation, the DNA was resuspended in 10 mM Tris·HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA and sonicated 4 times for 30 sec at 0°C in a Branson sonifier (microtip, position 7). DNA was digested with RNase and incubated for 1 hr at 37°C with autodigested Pronase (200 μg/ml) in the presence of 0.2% SDS, extracted 4 times with chloropane and precipitated with ethanol. The DNA pellets were washed twice with 70% (v/v) ethanol, dried and dissolved in water. DNA was denatured by boiling for 15 min and stored at −20°C at a concentration of 10 mg/ml.

Synthesis of biotinylated oligonucleotide

Oligonucleotides were synthesized on a 380B DNA Synthesizer (Applied Biosystems). Biotinylated oligonucleotide bearing the PTF1 cognate sequence of the α-amylase gene (Amy 2-IV; ref. 13) was prepared as follows. The noncoding strand oligonucleotide was biotinylated at its 5′ end by using Aminolink II (Applied Biosystems) and the protocol provided by the supplier. The biotinylated oligonucleotide was purified on a sequencing gel and mixed with coding strand oligonucleotide at 1 mg/ml final DNA concentration. Oligonucleotides were annealed in 10 mM HEPES (pH 7.8), 10 mM $MgCl_2$ and 0.1 mM EDTA by heating for 5 min to 65°C followed by slow cooling to room temperature. Reannealed oligonucleotide was stored in aliquots at −20°C.

Purification of PTF1 by NLP

Purified nuclei were incubated in siliconized plastic tubes at a concentration of 10^8 nuclei/ml in binding buffer (12% glycerol, 60 mM KCl, 12 mM HEPES (pH 7.8), 0.12 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT and 0.1% Triton X-100). Biotinylated Amy 2-IV oligonucleotide was added directly to the nuclear suspension in at least 20-fold molar excess over PTF1 DNA-binding activity. Binding reactions were performed for 1 hr at 20°C in the presence of protease inhibitors and single-stranded *E.coli* DNA as non-

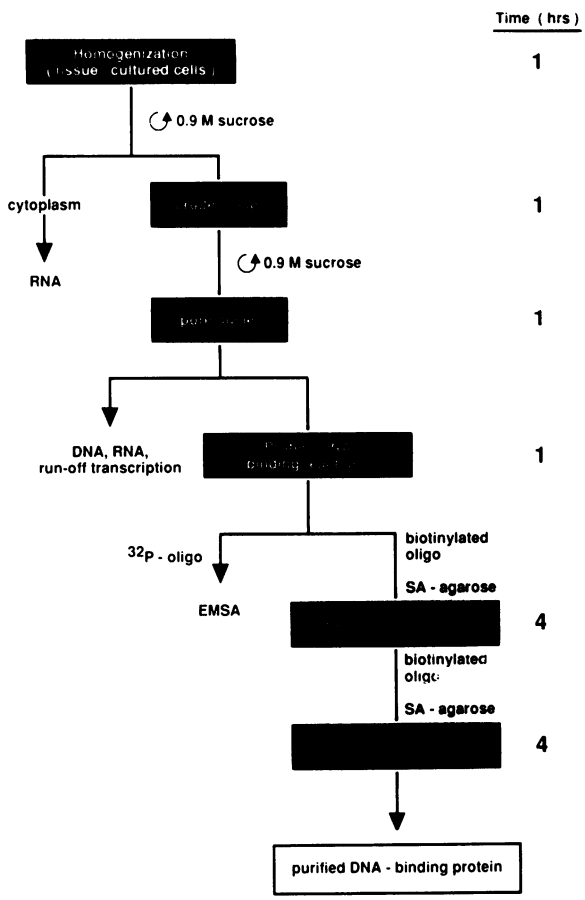

Figure 1. The rationale for the isolation of DNA-binding proteins from nuclei of tissues and cells in culture.

specific competitor in a 3000-fold (w/w) excess over the specific DNA sequence. After binding, nuclei were removed by centrifugation at 3 krpm and 0°C in the Sorvall HB-4 rotor. The postnuclear supernatant was centrifuged once more to remove debris that may clog up the DNA-affinity column. Streptavidin-agarose (SAA; 20 μl/μg oligonucleotide; GIBCO/BRL) was applied to a small column and washed, at 4°C, first with 10 volumes of binding buffer containing 2 M KCl, then with 5 volumes of binding buffer alone and finally with 5 volumes of binding buffer containing 200 μg/ml of single-stranded *E.coli* DNA. We routinely test each batch of SAA for its content of free streptavidin by EMSA using radiolabelled, biotinylated oligonucleotide. We have also found that some batches of SAA of certain suppliers retain proteins non-specifically. PTF1-DNA complexes were adsorbed onto the SAA beads by passing the postnuclear supernatant through the column at 4°C and a flow rate of 30 ml/hr. The column was then washed 10 times with 3 column volumes each of binding buffer containing 200 μg/ml of single stranded *E.coli* DNA and then 5 times with 2 column volumes each of binding buffer containing 150 mM KCl. PTF1 was eluted with binding buffer containing 450 mM KCl. The various column fractions were stored at −70°C. For the second round of DNA-affinity chromatography, eluted fractions from the first column were adjusted to 100 mM KCl by the addition of binding buffer containing a 20-fold molar excess of biotinylated oligonucleotide over PTF1 DNA-binding activitiy and a 300-fold molar excess of single-stranded *E.coli* DNA over the oligo-

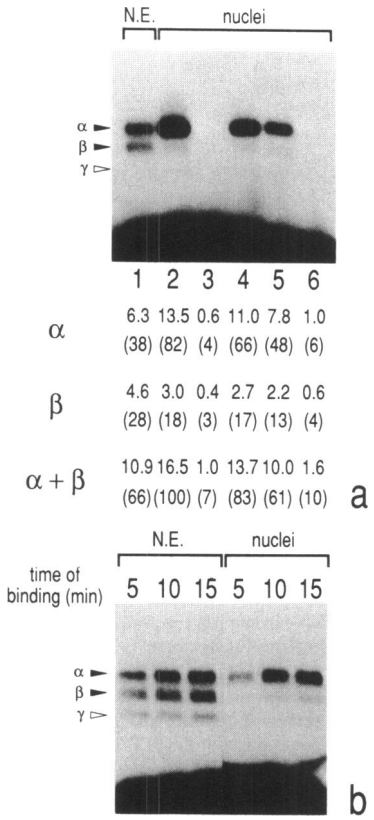

Figure 2. Quantitative analysis of PTF1 binding activity present in nuclei and N.E.. In (a), the binding activity of PTF1 in crude N.E. (ref. 7) and pure nuclei prepared from the same number (5×10^5) of AR42J cells is compared by EMSA. Binding reactions (20 μl) containing the PTF1 cognate sequence in form of a ^{32}P-labelled oligonucleotide (8.7×10^8 cpm/μg) were carried out for 30 min in the presence of non-specific competitor DNA. Binding reactions were then subjected to electrophoresis on a 2% agarose gel; and PTF1-DNA complexes were visualized by autoradiography. Lane 1, N.E.; lane 2, nuclei; lane 3, postnuclear supernatant of nuclei stored in buffer B; lane 4, nuclear pellet from lane 3; lane 5, postnuclear supernatant of nuclei from lane 2 after binding; lane 6, nuclear pellet from lane 5. α and β designate complexes containing different molecular forms of PTF1, and γ designates non-specific complex in this and all subsequent figures. α and β were quantitated by measuring Cerenkov radiation of dried down gel areas. Numbers are cpm$\times10^{-2}$. Numbers in parentheses are % PTF1 binding activity present in each reaction. The value for nuclei in lane 2 was taken arbitrarily as 100%. The gel of (b) compares the time profiles obtained with N.E. and nuclei for the binding of PTF1. Aliquots were removed from binding reactions at the times indicated and analysed by EMSA.

nucleotide. The binding reaction was carried out for 20 min at room temperature and was then applied to a newly prepared SAA column. All subsequent steps were performed as described above.

Electrophoretic mobility shift assay (EMSA)

Binding reactions for the detection of PTF1 in crude N.E. or purified nuclei were carried out according to ref. 14. Binding reactions for the detection of SP1 were done according to ref. 22. Protein-DNA complexes were visualized by electrophoresis on 2% agarose gels as described previously (12).

Other methods

AR42J rat acinar pancreatic cells were cultured as described by ref. 12. UV-crosslinking experiments with DNA probes containing azido deoxyuridine (N_3·dU) were done according to ref. 13. SDS-PAGE of proteins was performed as described by ref. 23 and silver staining of proteins in gels according to ref. 24.

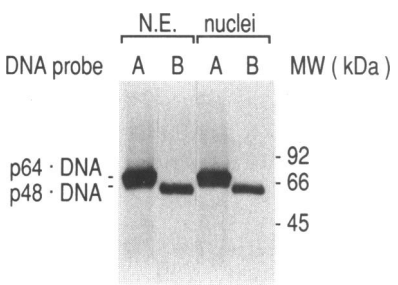

Figure 3. UV-crosslinking of PTF1 to N_3·dU-substituted cognate DNA. N_3·dU-substituted DNA probes A and B, which detect p64 and p48, respectively, were added separately either to purified nuclei or to N.E. prepared from pancreatic AR42J cells. Binding reactions were carried out for 30 min and were then exposed to short wave UV-light for crosslinking of PTF1 to DNA. The samples containing N.E. were applied directly to a 10% SDS-polyacrylamide gel. The samples containing nuclei were centrifuged first to remove nuclei and were then loaded onto the gel in parallel with commercial protein molecular weight standards (MW). p64·DNA and p48·DNA designate complexes containing the DNA-binding subunits of PTF1 crosslinked to DNA (ref. 13).

RESULTS AND DISCUSSION

The rationale for the purification of DNA-binding proteins is shown in the flow diagram of Fig. 1. It is based on the key observation that DNA-binding proteins are quantitatively recovered by incubating purified nuclei directly with oligonucleotide probes without preparing a N.E. first (for details see Materials and Methods). This nuclear leakage procedure (NLP) has several advantages over conventional methods. First, incubation of nuclei is carried out in binding buffer under conditions that are optimal for the DNA-binding activity of interest as determined by electrophoretic mobility shift assay (EMSA). A precise titration of non-specific DNA competitor in the binding reaction is crucial for a good enrichment of sequence-specific DNA-binding proteins. (Whenever possible, single-stranded rather than double-stranded DNA should be used since the former added at high concentration to the binding reaction does not affect sequence-specific protein-DNA interactions but efficiently competes for the binding of high abundance proteins having a low affinity for specific DNA.) Second, proteolytic degradation is minimized by i) using purified rather than crude nuclei, ii) the use of specific and non-specific protease inhibitors and iii) the binding of protein to the DNA early during purification.

Here we compare the quality and yield of a DNA-binding protein purified from nuclei of cells in culture using either the protocol of Fig. 1 or the commonly used N.E. procedure (NEP) of ref. 7. We have chosen PTF1 as a representative example of a DNA-binding protein. PTF1 is a pancreas-specific transcription factor that accumulates to only about 2000 copies per cell. It contacts the bipartite cognate DNA through two different DNA-binding subunits, p64 and p48 (12, 13). In addition, PTF1 contains a non-DNA-binding subunit, p75, that is required for its translocation from the cytoplasm to the nucleus (14). The various subunits exhibit differential sensitivity to proteolytic degradation with p64 being the most resistant, and p75 the most labile protein (data not shown). The EMSA of Fig. 2a shows

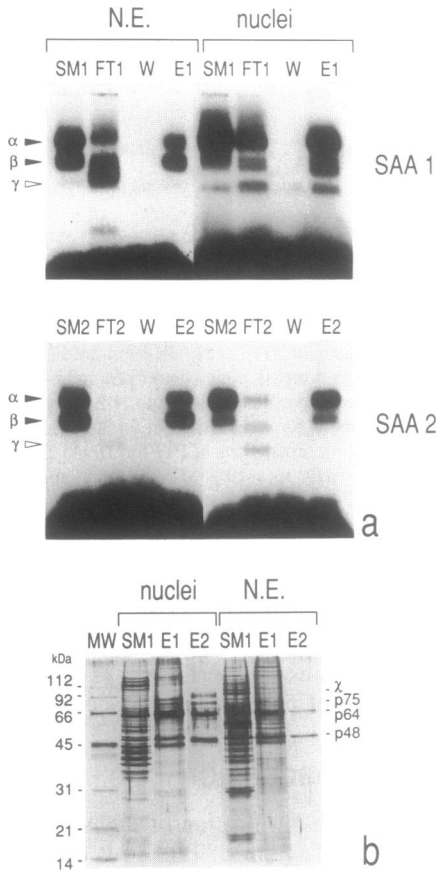

Figure 4. The purification of PTF1 by NEP and NLP. PTF1 was purified in parallel by NEP and NLP from a similar number of AR42J cell nuclei. The purification on SAA columns was monitored by EMSA (a) and PAGE (b). Analytical binding reactions for EMSA contain appropriate dilutions of the material applied to SAA columns. The PTF1 binding activity visualized by EMSA thus reflects the amount of PTF1 protein in each column fraction relative to that present in the starting material. Binding reactions containing SM (starting material) and FT (flow-through material) were carried out in the presence, and those containing W (combined material from column washes) and E (eluted material) in the absence of single-stranded DNA competitor. Protein in (b) was visualized by silver staining after electrophoresis on a 15% SDS-polyacrylamide gel. The lanes of the gel showing SM 1 of N.E. and nuclei contain each the amount of protein present in about 10^5 cell equivalents. The lanes showing E contain each about 40 ng of PTF1 DNA-binding subunits. The positions of PTF1 subunits p75, p64 and p48 are indicated and were determined in comparison to commercial protein molecular weight standards (MW). χ designates a protein that co-purifies with PTF1.

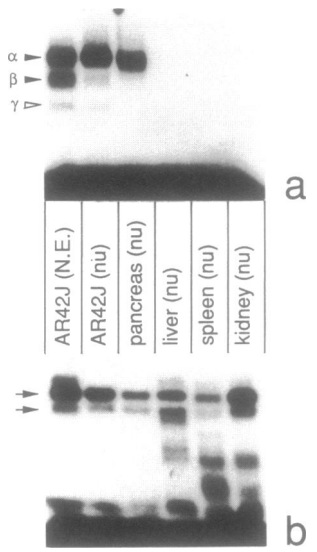

Figure 5. Isolation of DNA-binding proteins from purified nuclei of animal tissues. Nuclei were purified from tissues as described in Materials and Methods. Binding reactionse containing 2×10^5 nuclei (nu) each (or N.E. from the same number of nuclei) and radiolabelled oligonucleotides for the detection of transcription factors PTF1 (a) and SP1 (b), respectively, were analysed by EMSA. The SP1 DNA probe contains the SP1 binding site of the HSVtk gene promoter (22). The two complexes in (b) designated by arrows are assumed to contain SP1 since they were competed with a 100-fold molar excess of unlabelled specific, but not by non-specific oligonucleotide (data not shown).

Table 1. Quantitation of PTF1 purified by NLP and NEP.

Column	Fraction*	PTF1 (ng (%))† NLP		NEP	
SAA 1	SM 1	232	(100)	275	(100)
	FT 1‡	67	(29)	74	(27)
	W	< 1	(<1)	< 1	(<1)
	E 1 (=SM 2)	129	(56)	129	(47)
SAA 2	FT 2‡	9	(4)	7	(3)
	W	< 1	(<1)	< 1	(<1)
	E 2	83	(36)	98	(36)

* The designation of column fractions is that of Fig. 4. SM 1 is about 10^9 nuclei.
† The amount of PTF1 was determined indirectly from the data of Fig. 4a. The gel was dried down onto DEAE-paper and slices containing α and β were counted separately for each binding reaction by Cerenkov radiation. Background was determined by counting a blank area of the gel and was subtracted from α and β cpm. The values shown were calculated from the sum of α and β cpm. 1 ng of PTF1 (p64·p48 heterodimer) binds 4.8×10^5 cpm of ^{32}P-labelled Amy 2-IV oligonucleotide (8.7×10^8 cpm/μg).
‡ PTF1 binding in FT is a minimal estimate since only free but not DNA-bound protein is detected.

that NLP yields about 30% more PTF1 binding activity than NEP from a comparable number of nuclei. PTF1 prepared by NLP is of superior quality as judged from the stoichiometry of α and β forms of the factor, with α to β ratios of about 4:1 for NLP and 1.5:1 for NEP. α is the nuclear form of the factor while most of β is generated from α by proteolytic break-down of p75 during preparation. (β which is also a true cytoplasmic form of the factor constitutes less than 1% of total PTF1 binding activity of the cell.) PTF1 binding activity is almost quantitatively recovered in the post-nuclear supernatant after incubation of nuclei with radiolabelled oligonucleotide. Only a small amount of factor leaks from nuclei in the buffer used for storage. The time required for complete leakage of PTF1 from nuclei diluted into binding buffer is about 10 min (Fig. 2b). DNA-binding proteins may be visualized indirectly at this stage by UV-crosslinking to DNA probes containing, e.g., azido deoxyuridine (N_3·dU). Fig. 3 shows the results of an experiment in which N_3·dU-substituted oligonucleotides were incubated with nuclei or N.E. for the binding of PTF1. Analysis by PAGE demonstrates that crosslinking of PTF1 to DNA occurs as efficiently in binding reactions containing nuclei rather than N.E.

For purification of PTF1, we used a modification of the protocol of ref. 15. The PTF1 cognate sequence in form of a biotinylated oligonucleotide was added, either directly to nuclei diluted into binding buffer, or to N.E. made from a similar number of nuclei. The preparative binding reactions were allowed

to go to completion and DNA-bound PTF1 was adsorbed onto SAA beads. Two alternative approaches may be used for the binding of protein-DNA complex to SAA, adsorption in batch or on a preformed column. In our hands, the addition of SAA directly to the binding reaction improves the purity while chromatography on a preformed column increases the yield of protein. The data presented in this paper have all been obtained by column adsorption.

The results from EMSA show that NLP and NEP yield comparable amounts of PTF1 upon DNA-affinity chromatography (Fig. 4a). The yield is typically about 50% of input PTF1 binding activity for the first chromatography cycle (Table 1). PAGE analysis shows that material prepared by NLP is more enriched for PTF1 than that obtained by NEP (Fig. 4b). The reason for this is presumably the lower complexity of proteins in low salt washes of pure nuclei (compare SM 1 fractions of Fig. 4b). A second cycle of DNA-affinity chromatography is then carried out as a final purification step. For this, material eluted from the first column is diluted into binding buffer and processed as described for first cycle. The second column yields usually between 60 and 80% of PTF1 eluted from the first column (Fig. 4a and Table 1). Final recovery is thus greater than 30% of the binding activity present in the starting material. After two rounds of DNA-affinity chromatography, the purity of PTF1 obtained by the two procedures is comparable as judged by PA-GE (Fig. 4b). However, NEP yields reproducibly less p75 than NLP since a portion of p75 is proteolysed during preparation of N.E. (see, e.g., Figs. 2a and 4a).

In order to test whether NLP would also be useful for the isolation of DNA-binding proteins from tissues, we prepared nuclei from pancreas, liver, spleen and kidney. Purified nuclei were then incubated in separate reactions with radiolabelled oligonucleotides bearing the binding sites for PTF1 and the ubiquitous transcription factor SP1 (16), respectively; and protein-DNA complexes were analysed by EMSA. Fig. 5 shows that both DNA-binding proteins leak from tissue nuclei upon incubation in the respective binding buffers. As expected, PTF1 DNA-binding activity is restricted to pancreatic tissue while SP1 binding is detected in all tissues examined. The fact that PTF1 released from purified pancreatic nuclei is mostly α indicates that little proteolysis occurs during nuclear preparation from a tissue that is a major source for serine proteases. The quality of PTF1 from pancreatic nuclei is even superior to that of PTF1 from pancreatic cells in culture as deduced from the α to β ratio (compare Figs. 2a and 5). This may be due to the absence of detergent during the first homogenization step. Nonidet-P40, which is used for the lysis of cells in culture, is known to release proteolytic activities associated with cellular organelles. In addition to PTF1 and SP1, various other DNA-binding proteins including transcription factors AP-4 (17), NF1 (18) and the yeast scaffold binding protein RAP1 (19; J.F.-X. Hofmann, personal communication) have been successfully isolated using NLP (data not shown). We believe, therefore, that this method will prove generally useful for the isolation of DNA-binding proteins from a variety of tissues and cultured cells of different origins.

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