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High-resolution gel filtration of the ecdysteroid receptor–DNA complex —an alternative to the electrophoretic mobility shift assay

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ABSTRACT

The mobility shift assay is a well established method for proving binding of protein to DNA. However, this method depends on the stability of the protein–DNA complex during the electrophoretic process. Ecdysteroid receptor shows a strong tendency to aggregate under low-salt conditions of electrophoresis to a non DNA-binding form. We have developed a high-resolution gel filtration method which allows the interaction of ecdysteroid receptor with specific DNA sequences to be studied. The method seems to be generally applicable. It does not depend on the availability of a purified protein. Crude preparations could be used to characterize the stoichiometry and the molecular parameters of the complexes formed between DNA and DNA-binding proteins.

INTRODUCTION

Protein–DNA interactions are essential steps in many cellular functions. To study the binding of proteins to DNA the electrophoretic mobility shift assay is frequently used [1,2]. In this assay, protein–DNA complexes can be separated from free DNA by electrophoresis. The method depends on the ability of the electrophoretic system to resolve the reaction components and on the stability of the complexes during the separation process [2]. To obtain clearly interpretable results, highly purified protein samples are necessary for most applications. In the case of vertebrate steroid hormone receptors the method has been used very successfully to identify and characterize DNA sequences which specifically bind the hormone receptor [3] and are called hormone-responsive elements (HREs).

20-Hydroxy-ecdysone, found in invertebrates, is involved in the regulation of moults and the pupation of insects [4]. Owing to the lack of purified ecdysteroid receptor (EcdR) it was not yet possible to define clearly DNA sequences responsible for specific binding of EcdR (ecdysteroid responsive elements; EcdREs) [5,6]. In addition, EcdR aggregates under the low-salt condition of electrophoresis to a high-molecular-mass form which disturbs the mobility shift assay [7]. Gel filtration on Superose 6HR offers and alternative as the elution buffer can be freely chosen independent of the reaction conditions.

Based on the advantages of high-resolution gel chromatography, we have developed a method with which the specific interaction between EcdR and EcdREs can be studied in crude extracts. The method allows the characterization in terms of stoichiometry and molecular parameters of the protein–DNA complex and can be applied to other DNA binding proteins.

EXPERIMENTAL

Preparation of nuclear extracts and labelling with [^{3}H]ponasterone A

Nuclear extracts were prepared from embryos of an Oregon R stock of *Drosophila melanogaster* as described previously [8]. [^{3}H]Ponasterone A ([^{3}H]PonA) labelling of nuclear extract was routinely done by 30-min incubation with 40 n*M* [^{3}H]PonA at 25°C. The [^{3}H]PonA–EcdR concentration was determined using PD 10 column chromatography [8].

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Oligonucleotides

Double-stranded (ds) oligonucleotides were obtained by annealing of appropriate strands synthesized with a Gene Assembler (Pharmacia) and purified as recommended by the manufacturer. Oligonucleotide concentrations were determined spectrophotometrically assuming 37 μg of DNA per A_{260} unit for single-stranded oligonucleotides and 50 μg for double-stranded oligonucleotides [9]. The hsp27 ds-oligonucleotide (see Fig. 1), which according to Riddihough and Pelham [11] should contain an EcdRE, was ^{32}P labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase [13] and unincorporated [γ-^{32}P]ATP was removed using an NAP10 column (Pharmacia).

Gel filtration of [^{3}H]PonA–EcdR complexes

The nuclear extract was labelled with [^{3}H]PonA as described above. Excess of [^{3}H]PonA was removed using PD10 columns (Pharmacia) equilibrated with buffer A [20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)–KOH, 100 m*M* KCl–1 m*M* dithiothreitol–2 m*M* KH_2PO_4–1 m*M* EDTA, 10% (v/v) glycerol, pH 7.6 at 20°C]. Samples of 0.5 ml containing 0.42 ± 0.05 pmol of [^{3}H]PonA–EcdR were incubated for 30 min with 0.0–25 μg of poly(dI–dC) and subsequently for 30 min with 0.0–0.9 pmol of ^{32}P-labelled hsp27 ds-oligonucleotide on ice as indicated in the legends.

Gel filtration of the samples was carried out in a Pharmacia FPLC apparatus equipped with a Superose 6HR column equilibrated with buffer A. The flow-rate was 0.15 ml/min and 0.25-ml fractions were collected. In each fraction ^{3}H and ^{32}P radioactivities were determined as described below. Except where stated otherwise, the column was washed after each run with 3 *M* KCl solution, re-equilibrated with buffer A and coated by chromatography of 500 μl of a sample containing coating proteins as described in Fig. 2.

The column was calibrated with bovine thyroglobulin (Sigma) (Stokes radius, R_s = 8.6 nm; molecular mass, m = 670 000 dalton), bovine serum albumin (Sigma) (R_s = 3.6 nm, m = 67 000 dalton), ovalbumin (Sigma) (R_s = 2.9 nm, m = 45 000 dalton) and myoglobin (Serva) (R_s = 2.0 nm, m = 17 800 dalton). The void volume was determined by filtration of dextran blue (Pharmacia).

Radioactivity measurement

Samples were mixed with 7 ml of Quickszint 2000 (Zinsser). Radioactivity was measured in a LKB liquid scintillation spectrometer with an efficiency of 40% for ^{3}H and 100% for ^{32}P. Both radioactivities were corrected for ^{32}P spillover and for radioactive decay of the labelled DNA assuming a ^{32}P half-life of 14.3 days.

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hsp27    ctAGACAAGGGTTCAATGCACTTGTCCAtcg
         gaTCTGTTCCCAAGTTACGTGAACAGGTagc

hsp27Δ   ctAGACAAGGGTTCA· · · ·ACTTGTCCAtcg
         gaTCTGTTCCCAAGT· · · ·TGAACAGGTagc
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Fig. 1. Structure of the synthetic ds-oligonucleotides used to study the specific interaction of [^{3}H]PonA-labelled EcdR with DNA. hsp27 represents a DNA fragment derived from the upstream region of the hsp27 gene [10], which was sufficient to confer 20-hydroxyecdysone inducibility on a heterologous gene [11]. Small letters represent nucleotides added to the original hsp27 sequence for cloning purposes. hsp27$_\Delta$ represents the sequence in which four bases from the hsp27 central palindrome were deleted [12].

RESULTS

Pretreatment of the chromatographic system

Handling of picomolar amounts of proteins or nucleic acids in chromatographic systems is sometimes difficult. Wall and resin surface effects can adversely affect the elution behaviour of the solutes. Also, low recoveries are not unusual. Such problems can only be overcome if the status of the column is carefully controlled. After each experiment the column has to be cleaned and active adsorptive centres must be covered with substances that show a minimum interaction with the chromatographic substrates. Such pretreatment of the column is a prerequisite for reproducible elution results.

The conditioning of the Superose 6HR column was started in all instances by applying 2 ml of 3 *M* NaCl solution and washing with one column volume of buffer A. To find the optimum conditions, a constant amount of preformed [^{3}H]PonA–EcdR–[^{32}P]hsp27 ds-oligonucleotide complexes were chromatographed (Fig. 2). Free [^{3}H]PonA eluted behind the column volume (V_t), indicating an interaction of the hormone with the gel matrix. Unbound [^{32}P]hsp27 ds-oligonucleotide appeared around

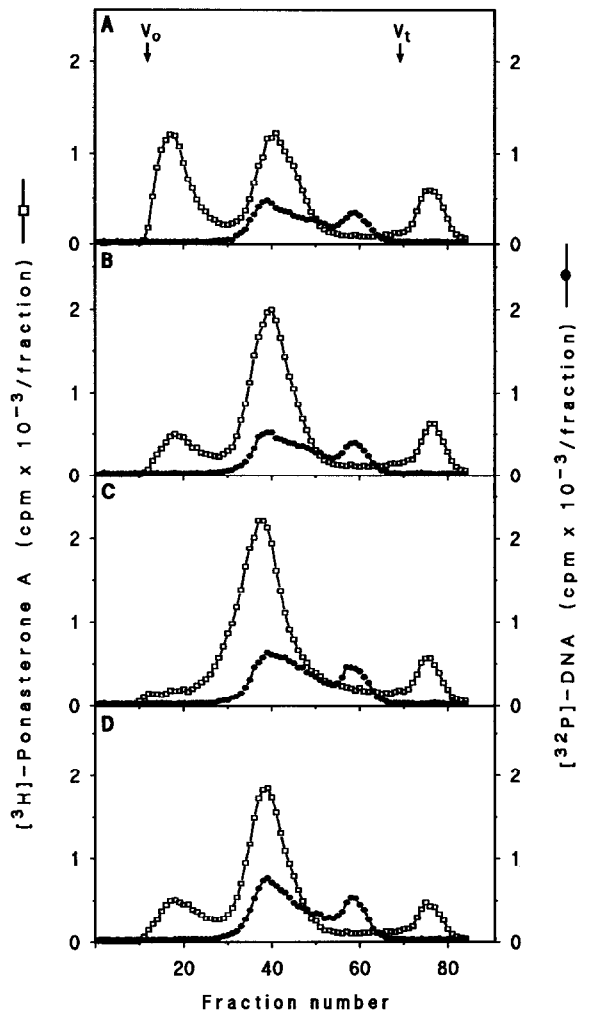

Fig. 2. Gel filtration analysis of specific [^{3}H]PonA–EcdR complex with ^{32}P-labelled hsp27 DNA. Effect of primary treatment of the Superose 6HR column. [^{3}H]PonA–EcdR was separated from excess of [^{3}H]PonA as described under Experimental. Samples of 0.5 ml containing *ca.* 0.42-pmol of [^{3}H]PonA–EcdR were incubated for 30 min with 0.25 μg of poly(dI–dC), followed by a 30-min incubation with 0.09 pmol of [^{32}P]hsp27 DNA. EcdR–DNA complexes were analysed on the Superose 6HR column which was prepared as follows: (A) the column was washed with 3 *M* KCl and equilibrated with buffer A; (B) the column was washed with 3 *M* KCl and after equilibration with buffer A coated with 2 mg of ovalbumin at a flow-rate of 0.15 ml/min; (C) the column prepared as described in (B) was used for chromatography of a nuclear extract sample containing [^{3}H]PonA–EcdR–DNA complexes and without additional preparation used for chromatography of a new sample; (D) the column was washed with 3 *M* KCl, equilibrated with buffer A and coated by chromatography of a 500-μl probe containing 5 mg of ovalbumin and 5 mg of γ-globulin; the flow-rate was 0.06 ml/min. V_0 = void volume of the column; V_t = total volume of the column.

fraction 59. Both elution positions were independent of the pretreatment of the column.

After only cleaning the column with NaCl the [^{3}H]PonA–EcdR eluted around fraction 41 (Fig. 2A). The peaks of the [^{3}H]PonA–EcdR and of the [^{32}P]hsp27 ds-oligonucleotide did not match and both peaks were strongly tailing, which may suggest an interaction between the constituents of the just-formed EcdR–DNA complexes and the column matrix. High-molecular-mass aggregates of [^{3}H]-PonA–EcdR which did not bind [^{32}P]hsp27 ds-oligonucleotide eluted just behind the void volume.

When the column was coated with a solution containing 2 mg of ovalbumin using a flow-rate of 0.15 ml/min (Fig. 2B), the yield of the [^{3}H]PonA–EcdR was drastically increased. However, the [^{3}H]PonA–EcdR and the [^{32}P]hsp27 peaks were still broad and tailing. The column was then used without a further 3 *M* NaCl wash, which means *de facto* a pretreatment with nuclear extract (Fig. 2C). Again, the yield of [^{3}H]PonA–EcdR was increased and the high-molecular-mass material brought a minimum, but now the peak of the protein-bound [^{32}P]hsp27 ds-nucleotide was strongly tailing and was significantly retarded.

It is known in the gel chromatography of large biomolecules that decreasing the column flow frequently results in better resolution. However, at the same time the yield of the chromatographic substrate is sometimes drastically decreased. This effect was used for a more efficient coating of the Superose 6HR column. During pretreatment with a solution of 5 mg of ovalbumin and 5 mg of γ-globulin the flow-rate was decreased to 0.06 ml/min (Fig. 2D), allowing the coating run to be finished overnight. Only under these conditions could good chromatographic yield of [^{3}H]PonA–EcdR and [^{32}P]hsp27 ds-oligonucleotide of over 80% be achieved and the [^{3}H]PonA–EcdR peak had symmetrical appearance. For all further experiments the column was conditioned according to Fig. 2D.

Effect of general competitor DNA

In these experiments crude nuclear extracts which contain many DNA-binding proteins in addition to the EcdR were used [12]. In the electrophoretic mobility shift assay, poly(dI–dC) is commonly used as a competitor to suppress the binding of such proteins [1,2]. The optimum amount of competitor

DNA had to be determined by titrating [^{3}H]PonA–EcdR–[^{32}P]hsp27 ds-oligonucleotide complexes with poly(dI–dC). [^{3}H]PonA–EcdR–[^{32}P]hsp27 ds-oligonucleotide complexes were chromatographed in the presence of 0, 0.25, 2.5 and 25 μg of poly(dI–dC) (Fig. 3). With 25 μg most of the [^{3}H]PonA–EcdR co-elutes with the poly(dI–dC) in the void volume of the column (Fig. 3D). Obviously in the presence of such a large excess of competitor the [^{3}H]PonA–EcdR binds to poly(dI–dC). With 0.25 μg of poly(dI–dC) the [^{32}P]hsp27 ds-oligonucleotide peak co-eluting with the [^{3}H]PonA–EcdR is still very asymmetric (Fig. 3B). This indicates that other nuclear proteins still bind to the [^{32}P]hsp27 ds-oligonucleotide and that such complexes are eluting in the region between peaks 2 and 3. A 2.5-μg amount seems to be optimum, resulting in symmetrical peaks of both the [^{3}H]PonA–EcdR and the [^{32}P]hsp27 ds-oligonucleotide.

All further experiments were done in the presence of 2.5 μg of poly(dI–dC).

Can DNA binding of EcdR be saturated?

To answer this question, increasing amounts of [^{32}P]hsp27 ds-oligonucleotide were added to the nuclear extracts containing EcdR. Molar ratios of [^{32}P]hsp27 ds-oligonucleotide to [^{3}H]PonA–EcdR of 0.20:1, 0.74:1, 1.5:1 and 2.1:1 were used. The elution diagrams are shown in Fig. 4. For information purposes the A_{280} profile is also given in Fig. 4A. This shows where the main protein material of the nuclear extract is eluted. DNA-binding protein(s) eluting under peak 3 in Fig. 4A–D cannot be saturated within the limits of [^{32}P]hsp27 ds-oligonucleotide applied. Integration of the ^{32}P peak 3 yields a linear dependence on the amount of [^{32}P]hsp27 ds-oligonucleotide added. In contrast, the [^{3}H]PonA–EcdR peak 2 is clearly saturable with [^{32}P]hsp27 ds-oligonucleotide. By integration of the ^{32}P and ^{3}H radioactivities, a maximum binding at a molar ratio of [^{32}P]hsp27 ds-oligonucleotide to [^{3}H]PonA–EcdR of 1:2 can be calculated [12].

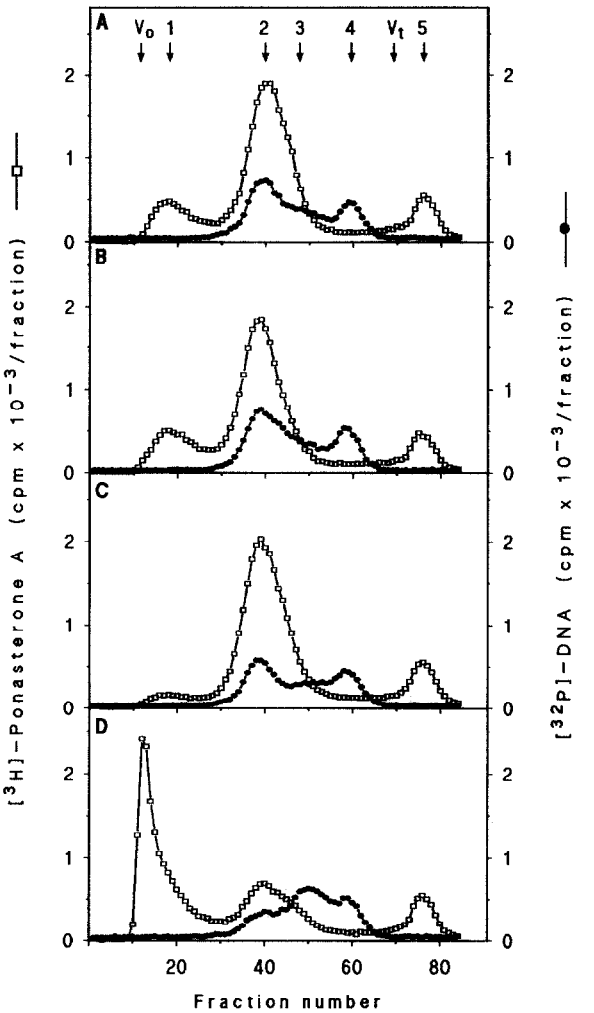

Fig. 3. Effect of poly(dI–dC) concentration of specific complex formation between [^{3}H]PonA–EcdR and ^{32}P-labelled hsp27 DNA. Samples of 0.5 ml containing *ca.* 0.42 pmol of [^{3}H]PonA–EcdR were incubated for 30 min wiht (A) 0, (B) 0.25, (C) 2.5 or (D) 25 μg of poly(dI–dC), followed by incubation with 0.09 pmol of [^{32}P]hsp27 DNA. EcdR–DNA complexes were analysed with the Superose 6HR column prepared as in Fig. 2D. Peak 1 corresponds to a high-molecular-mass form of [^{3}H]PonA–EcdR, 2 to the [^{3}H]PonA–EcdR–[^{32}P]hsp27 complexes, 3 to unspecific protein–[^{32}P]hsp27 DNA complexes, 4 to unbound [^{32}P]hsp27 DNA and 5 to unbound [^{3}H]PonA. Other symbols as in Fig. 2.

Molecular mass determination

Free [^{3}H]PonA–EcdR elutes as a broad peak tailing to the high-molecular-mass side (Fig. 5). From the peak maximum a molecular mass of 130 000 dalton can be calculated, as indicated in the inset in Fig. 5. This is in excellent agreement with earlier reports where the determination was based on sodium dodecyl sulphate–polyacrylamide gel electrophoresis [14]. In the presence of poly(dI–dC), the peak becomes more symmetrical and seems to be very slightly shifted to higher molecular mass. This

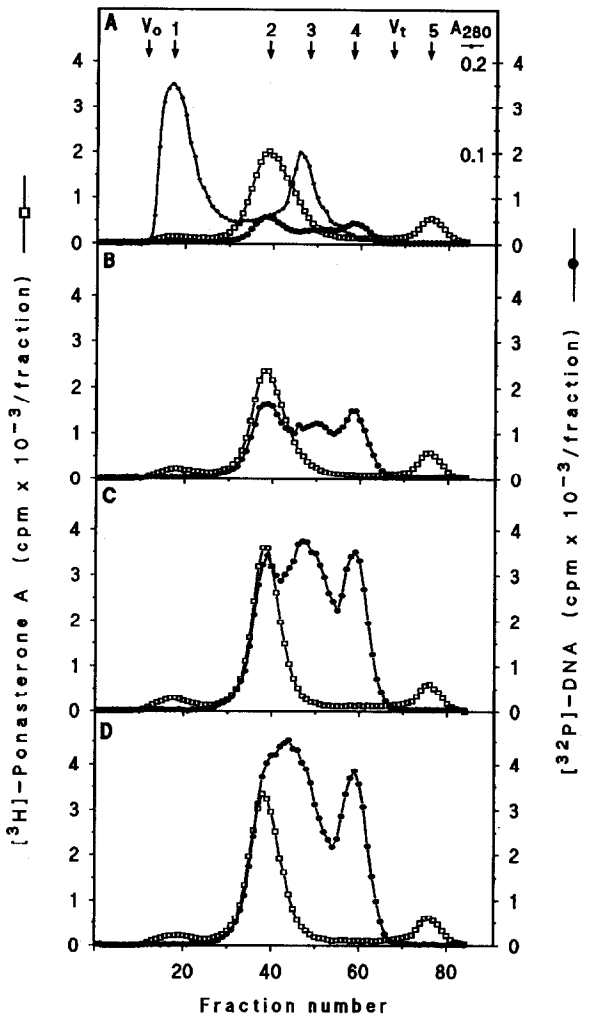

Fig. 4. Saturation of EcdR with hsp27 DNA. Samples of 0.5 ml containing *ca.* 0.42 pmol of [^{3}H]PonA–EcdR were incubated with 2.5 μg of poly(dI–dC) and then with (A) 0.09, (B) 0.31, (C) 0.61 or (D) 0.90 pmol of [^{32}P]hsp27 ds-oligonucleotide. Samples were analysed with the Superose 6HR column prepared as in Fig. 2D. Symbols as in Fig. 3.

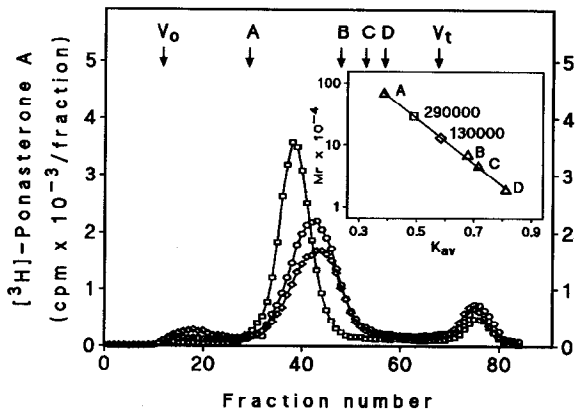

Fig. 5. Changes in the apparent molecular mass of [^{3}H]PonA–EcdR complexes on interaction with DNA. [^{3}H]PonA–EcdR complexes (0.42 pmol) were chromatographed on the Superose 6HR column (◇) after 60-min incubation, (○) after 60-min incubation with 2.5 μg of poly(dI–dC) or (□) after 30-min incubation with 2.5 μg of poly(dI–dC) followed by 30-min incubation with 0.90 pmol of hsp27 DNA. Arrows mark the elution of standard proteins (see Experimental). The column was prepared as in Fig. 2D. Inset: distribution coefficients (K_{av}) were calculated from elution volumes (V_e) according to Laurent and Killander [15]. Apparent molecular mass values for [^{3}H]PonA–EcdR (130 000 ± 15 000 dalton) and for [^{3}H]PonA–EcdR saturated with hsp27 ds-oligonucleotide (290 000 ± 30 000 dalton) were calculated by reference to a calibration graph.

indicates that under the influence of DNA, in this instance poly(dI–dC), the EcdR has some increased tendency to aggregate. During saturation with an excess of [^{32}P]hsp27 ds-oligonucleotide the appearance of the [^{30}H]PonA–EcdR is dramatically changed to a sharp peak. At the same time, the peak is shifted and the maximum now appears at a molecular mass of 290 000 dalton, demonstrating a complex of two molecules of EcdR and one molecule of hsp27 ds-oligonucleotide [12].

Competition by specific DNA

Assuming specific binding of the [^{32}P]hsp27 ds-oligonucleotide to the [^{3}H]PonA–EcdR, competition is provided by an excess of non-radioactive ds-oligonucleotide. This experiment is shown in Fig. 6A. The conditions are as in Fig. 4B except that a 6.7–fold excess of non-radioactive over [^{32}P]hsp27 ds-oligonucleotide was used. This excess is sufficient to move the main ^{32}P material away from the [^{3}H]PonA–EcdR peak. The [^{3}H]PonA–EcdR peak shows the typical sharp appearance of the nucleotide-saturated EcdR (see Fig. 5).

Such competition experiments can also be used to determine the binding of modified ds-oligonucleotides to the EcdR. An example is demonstrated in Fig. 6B. Again the conditions as in Fig. 4B were used. To preformed [^{3}H]PonA–EcdR–[^{32}P]hsp27 ds-oligonucleotide complexes a 6.7-fold excess of hsp27A ds-oligonucleotide (see Fig. 1) was added.

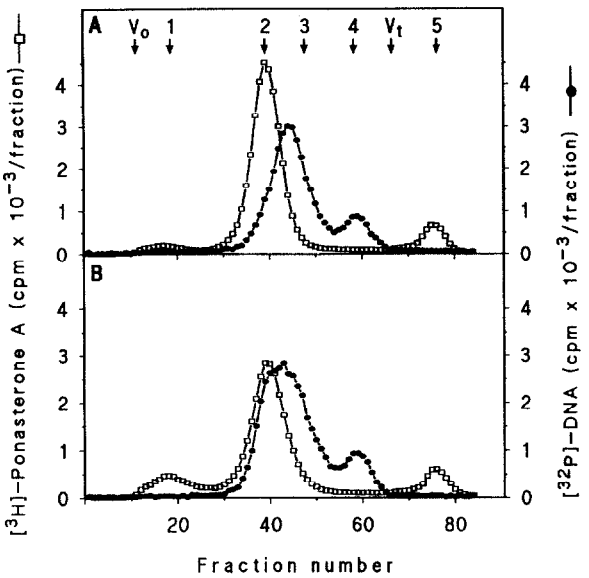

Fig. 6. Gel filtration analysis of the specificity of interaction between [^{3}H]PonA–EcdR and [^{32}P]hsp27 DNA fragment. (A) A 0.5-ml sample containing *ca.* 0.42 pnmol of [^{3}H]PonA–EcdR complexes was incubated for 30 min on ice with 2 pmol of unlabelled hsp27 ds-oligonucleotide. After additional incubation with 0.30 pmol of [^{32}P]hsp27 DNA the gel filtration analysis of the competition between ^{32}P-labelled and unlabelled hsp27 DNA for binding of [^{3}H]PonA–EcdR was done using the Superose 6HR column pretreated as in Fig. 2D. (B) The same as in (A), but 2 pmol of the unlabelled $hsp27_A$ ds-oligonucleotide (see Fig. 1) were used. Symbols as in Fig. 3.

Obviously the modified ds-oligonucleotide can only partially compete with the original.

DISCUSSION

The basic conditions of a gel filtration assay have been worked out which can be used for the identification of DNA sequences tightly bound by proteins.

If a specific detection system for the protein is available, crude extracts can be studied. In our case a ^{3}H-labelled ligand of the protein together with the [^{32}P]hsp27 ds-oligonucleotide made the fractions of the column effluent directly amenable to liquid scintillation counting, but all specific detection systems usually used in column chromatography can in principle be used.

The DNA binding assay depends on the ability of the gel filtration column to resolve the DNA–protein complex from free protein and from the unbound ds-oligonucleotide. Therefore, the size of the ds-oligonucleotide must be carefully chosen so that a separation of the complex within the limits of gel filtration is possible.

The assay also is dependent on the stability of the protein–DNA complex during the chromatographic separation process. Here the gel filtration system shows great advantages as the buffers can be chosen fairly freely. All kinds of additives which stabilize the reaction products or cofactors can be incorporated into the solvents. The addition of proteinase or DNase inhibitors may be crucial if crude extracts are used which frequently contain degrading enzymes. In contrast to electrophoresis, the buffer constituents do not influence the temperature during separation and optimum temperature can be easily maintained.

High-performance gel filtration columns, like the Superose 6HR, can be operated fairly fast without losing resolution. In the experiments discussed here the EcdR–hsp27 ds-oligonucleotide complex was eluted after *ca.* 90 min. This might be important for such systems where the protein–DNA or the protein–ligand complex has a short half-life.

Additional information on the protein–DNA complex is directly available from the elution diagram. With the help of saturation experiments the stoichiometry and specificity of the complex can be studied. As the elution positions in a modern chromatographic system are extremely reproducible, the molecular mass of the complex and any molecular mass changes resulting from interaction of a protein with specific DNA can be determined.

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