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A RAPID AFFINITY METHOD FOR ISOLATION AND CHARACTERIZATION OF SEQUENCE SPECIFIC DNA BINDING FACTOR

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SUMMARY- An affinity chromatography technique using avidin monomer and biotin interaction was used for isolating a sequence specific DNA binding factor. This approach was used for purifying a transcription factor that binds to 152 to 160 base pairs upstream of the transcription start site of adenovirus IVa_2 promoter. A 432 bp IVa_2 promoter fragment was isolated and was incorporated with biotin at the 5' end using Bio-11-dUTP and Klenow fragment of DNA pol I, it was then bound with factor in vitro in reactions containing hela cell extract in optimized conditions. After incubation, reactions were loaded onto an avidin monomer column. Adenovirus IVa_2 transcription factor was purified by 0.3M, 0.6M Nacl in binding buffer, band retardation assays demonstrated a 12,000 fold purification of factor was obtained.

INTRODUCTION- Recent investigations in the area of gene regulation have indicated the presence of a variety of factors which are involved during initiation of transcription or termination processes. Foot print analysis (1) and band competition assays using gel electrophoresis (2,3) have shown the presence of sequence specific DNA binding elements. A 17 bp region of adenovirus major late promoter that is involved in efficient transcription in vivo and in vitro has been observed to be protected by a factor (4). Reports from this laboratory had shown the presence of a protein factor interacting with the distal domain of adenovirus IVa_2 promoter and involved with transcription (5). We have used the avidin-biotin affinity system to purify sequence specific DNA binding factors. The introduction of biotinylated nucleotides has allowed the use of DNA probes for detection of specific DNA sequences (6-8).

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Purification of a biotin containing enzyme from crude extract was demonstrated on a monomeric avidin sepharose affinity column (9). Recently, DNA protein complex in the form of intact 11S nucleosome has been isolated using a similar approach (10). We have demonstrated the use of this system in the purification of a adenovirus transcription factor that had been observed to bind at 142 to 152 bp upstream of the transcription start site of adenovirus IVa_2 promoter.

MATERIALS AND METHODS - Adenovirus IVa_2 DNA was prepared by digesting pφ4 plasmid with EcoRI and HindIII and isolating a 433 bp DNA fragment by electroelution from agarose gels. The DNA fragment was then subjected to phenol extraction and chloroform treatment followed by ethanol precipitation. The DNA was treated with alkaline phosphatase followed by 5' end labelling using T_4 polynucleotide kinase and $[\gamma\text{-}^{32}P]$ ATP as described (11). Labelled or cold fragment was filled in with biotin in 25 µl reactions of 500 mM Tris, pH 7.5; 50 mM $MgCl_2$; 1mM DTT in the presence of 1 mM each of dATP, dCTP, dGTP, and 0.05 mM Bio-11-dUTP. 2 µl Klenow enzyme (6 U/µl) was added last and incubated at 37°C for 30 min. Biotinylated DNA was separated from unincorporated nucleotides by chromatography through 1 ml spin columns of Sephadex G-50 equilibrated in binding buffer.

Salmon sperm DNA was sonicated, extracted two times with phenol once with chloroform and precipitated with ethanol. Hela cell extract was prepared according to the method of Manley et al (12) with modifications as described (13). The cell extract was dialysed in binding buffer before use in binding reactions.

The conditions for DNA binding for this factor were optimized in this laboratory. A typical assay in a total volume of 25 µl contained: 10 mM Tris, pH 7.5; 150 mM Nacl; 1 mM EDTA; 10 mM 2-mercaptoethanol; 4% glycerol containing 2 µg salmon sperm DNA with 1-2 ng of cold or ^{32}P labelled biotinylated 432 bp IVa_2 promoter fragment. All the components except DNAs were part of the binding buffer. About 10 µg of hela cell extract was added in last and incubated for 30 min at room temperature.

The binding reactions were loaded directly onto regenerated avidin monomer agarose (Sigma) column and incubated for 1 hr while recycling the flow through 3-4 times. Avidin columns were washed with ten volumes of binding buffer. Protein was eluted sequentially by three column volumes each of binding buffer having 0.3M, 0.6M and 1.0M Nacl. The entire process was done at 4°C. This column was designated as B^+. DNA binding activity of fractions were determined using band retardation assays in which reactions were loaded onto low ionic strength polyacrylamide gels (4%) under conditions as described (14). Active fractions were dialysed, iodinated and subjected to SDS-polyacrylamide gel electrophoresis (15) for analysis of protein components. The column was regenerated after every use as described by supplier. A control column (B^-) was also set up under similar conditions where IVa_2 DNA was used without biotinylation. Rest of the conditions eg. DNA binding, affinity chromatography, gel analysis were maintained same as that for B^+ column. Gels were dried and autoradiographed. Elution of binding activity was compared in various fold diluted hela cell extract with purified B^+ fraction having maximum binding activity. Protein concentrations were determined in hela cell extract and active B^+ fraction having equal band densities. Estimations of protein in hela cell extract were performed by bio-rad dye method and trichloroacetic acid counts were used to determine protein concentration in ^{125}I-labelled purified B^+ fraction taking bovine serum albumin as standard that was iodinated under similar conditions.

Iodinations of affinity purified fractions having maximum binding activity was done as described : Three ml fraction taken from B^+ or B^- columns was concentrated to 70-100 µl and added with 5 µg of bovine serum albumin as carrier. Proteins were precipitated in 10% trichloro acetic acid, washed with cold acetone and finally dissolved in 20 µl of 100 mM borate buffer, pH 7.8. Proteins were iodinated by 150 µCi of [^{125}I]-Bolton Hunter Reagent (2463 Ci/mmol). After 30 min incubation at 4°C, 100 µl of 200 mM glycine in borate buffer was added. The reaction mixture was incubated for 30 min and dialysed against borate buffer to remove unreacted reagent. Samples were analysed by SDS-polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION- The introduction of biotinylated nucleotides (16)has enabled the utilization of avidin-biotin affinity system in detecting specific DNA sequences by using biotinylated DNA as hybridization probes (6-8) or in isolating 11S nucleosomes (10). Since avidin-biotin is tightly bound and irreversible, use of monomeric avidin, streptavidin or iminobiotin, which shows lower affinity and are reversible in binding, appeared more useful. We employed this system for purifying a factor that had been shown to interact with 152 to 162 bp upstream of adenovirus IVa_2 transcription start site and necessary for efficient transcription (5). EcoRI digested 432 bp adenovirus IVa_2 promoter fragment was incorporated with biotin using bio-11-dUTP and Klenow fragment of DNA pol I. Biotinylated DNA moved slightly slower when electrophoresed with non-biotinylated DNA in 3% agarose gel. Biotinylated DNA was used for binding with factor present in hela cell extract. Previous reports from this laboratory have shown the optimum binding conditions that depend on concentrations of Nacl (150 mM) and a competitor DNA (80 µg/ml). Variations in both parameters affected the binding of protein to DNA. The binding mixture was loaded and passed through the column as described in Methods. The reaction contained 5-10% of 5' end labelled and biotinylated DNA to monitor DNA binding to column matrix. On passing the binding mixture through affinity column 50-60% of DNA was retained. The protein was eluted using different salt concentration (0.3M, 0.6M, 1.0M Nacl) in binding buffer. The IVa_2 promoter binding activity was assayed in each of these fractions by gel retardation technique. Maximum activity of factor eluted in 0.6M fraction followed by 0.3M fraction (Fig. 1b). Gel retardation assays demonstrated only two bands were present in 0.6M fraction as compared to five to six bands present when hela cell extract was bound with IVa_2 promoter (Fig. 1a). These results indicated that IVa_2 promoter binding factor interacts with DNA sequences in combination with loosely bound secondary factors in hela extract which might be separated during purification. Alternatively it may also be possible that the same sequences are recognized by factors of different

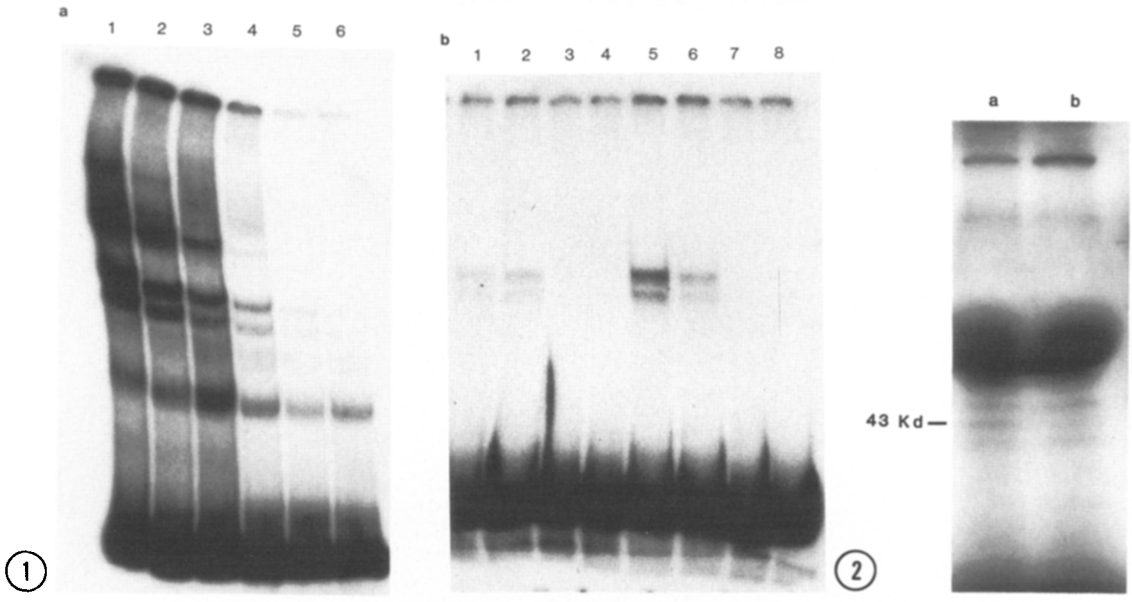

Figure 1. a-Band retardation assay of diluted hela cell extract. Binding assay was done as described in methods. 1, Undiluted; 2, Two-fold; 3, Five-fold; 4, Ten-fold; 5, Twenty five-fold; 6, Fifty-fold.

b-Band retardation assay of purified fractions eluted with binding buffer having: 1-3, 0.3M Nacl; 4-6, 0.6M Nacl; 7-8, 1.0M Nacl. Affinity chromatography and binding assay (in a total of 50 µl reaction) were done as described in methods.

Figure 2. Purification of adenovirus IVa_2 promoter binding factor by avidin monomer-biotin chromatography. Binding reaction having 6,500 µg of total hela cell protein was loaded on 3 ml column as described in methods. Each fraction was eluted with one column volume of binding buffer having different salt concentrations. Fractions containing maximum DNA binding activity from B^+ column and respective fraction from B^- column were iodinated and analysed for protein components by SDS-polyacrylamide gel electrophoresis. a-Fraction (0.6M Nacl) from B^+ column having IVa_2 DNA binding activity. b-Fraction (0.6M Nacl) from B^- column having no IVa_2 activity.

molecular weight yielding different bands. In a second set of experiments that were done under similar conditions and described as control (B^-) experiment the non biotinylated IVa_2 promoter was used for binding with hela cell extract and then passed through an avidin monomer column. As expected the DNA bound with factor was not retained. When assayed the 0.3-1.0M fractions did not show any binding activity. 0.6M fractions from B^+ and B^- columns were iodinated using Bolton Hunter Reagent as described and subjected to SDS-polyacrylamide gel electrophoresis for the comparison of protein components. It was observed that a 43 kd factor that was only present in B^+ fraction and not in the B^- fraction from the control experiment (Fig. 2).

TABLE 1. Purification of adenovirus IVa_2 promoter binding factor

Step*	Total protein (µg)	Bound DNA (pmol)	Specific activity (pmol bound/mg protein)	Purification fold
1	6420	3.124	0.486	-
2	0.042	0.257	6120	12,600

* 1. Hela cell extract, 2. After affinity purification

Comparison of remaining protein bands in B^+ and B^- lanes indicated that these are not specific to IVa_2 promoter as they co-purified in the B^- column which did not show any binding activity with IVa_2 promoter DNA. It appeared therefore, that these non specific proteins are retained because of interaction with agarose and are eluted with IVa_2 binding factor. Gel retardation assays of various fold diluted hela cell extract and purified B^+ fractions were performed. Protein concentrations of 0.6M B^+ fraction and diluted hela cell extracts having the same values after densitometric scanning of the gel retarded bands, were determined. It was observed that total 3.1 pmol of IVa_2 DNA was bound with factor present in hela cell extract, while after elution 0.25 pmol IVa_2 DNA was required to bind with purified factor indicating 8% yield of factor was obtained. Determination of specific activities yielded 12,600 fold purification of factor (Table 1) was achieved. Therefore this method can efficiently be utilized in isolation and purification of DNA binding factors. Significantly greater amounts of non specific proteins can be removed in relatively simpler step. Purified factors will contribute significant information in studying genes responsible for coding these factors.

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