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Bi-column method for purification of transcription factors

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Abstract

A novel bi-column method for purifying transcription factors, using two different columns and two different elution strategies is described. Lac repressor elutes at lower heparin concentrations from a lower affinity lactose operator1 (Op1)-Sepharose column than from a higher affinity column containing the same sequence with a T18:A18 tail ($Op1T_{18}$). A bi-column method was developed in which lac repressor fusion protein is eluted from the Op1-Sepharose with a low heparin concentration and trapped on a $Op1T_{18}$-Sepharose column because of its higher affinity for the lac repressor protein. Elution of the latter column with buffer containing a high salt concentration gives significantly purer transcription factor than the conventionally used single column methods and removes residual heparin. Highly pure CAAT enhancer binding protein and the B3 transcription factor are also obtained by using variants of this bi-column method.

Keywords: Coupled columns; DNA; Proteins

1. Introduction

Purification and characterization of transcription factors and other DNA binding proteins such as DNA polymerases, and endo- and exonuclease is important. These proteins control important cellular processes such as regulating the transcription of genes, DNA replication, recombination, cell division and differentiation. Moreover these proteins are frequently present in cells in small amounts and hence their purification is challenging.

A typical purification for transcription factor such as Sp1 involved purification through five steps [1], the last being DNA affinity chromatography. All these required steps make this purification time consuming. Furthermore, since no purification step yields complete recovery, the overall yield is often low. DNA affinity chromatography offers the highest selectivity and hence is widely used for purification of DNA binding proteins. Improvements in DNA affinity chromatography could allow purification in a lesser number of steps and significantly reduce the efforts required.

DNA affinity columns are made by attaching either nonspecific [2] or specific [3] DNA sequences to a variety of supports such as cellulose, agarose, silica, etc., and has been discussed in more detail elsewhere [4,5]. Nonspecific DNA (e.g., salmon sperm or other fragmented genomic DNA) selects only for the property of DNA binding and hence nonspecific DNA columns are not selective for a particular protein. Specific columns on the other hand are highly selective and are made by coupling carefully designed sequences, which are specifically

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bound by the protein of interest. Although specific columns are highly selective, more than one protein can often bind to these columns and contaminate the protein of interest.

Several approaches to improving the purity obtained have been reported. These include repeated use of DNA affinity columns [6], using nonspecific and specific sequence DNA affinity columns in tandem [7], using multimeric (concatemeric) DNA columns [8], washing the columns with a nonspecific DNA sequence or pre-incubating extracts with nonspecific DNA sequences [1,5]. In spite of these efforts, homogenous protein is rarely obtained with DNA affinity chromatography as the only step and clearly new approaches are needed. We have previously shown that DNA binding proteins such as lac repressor and CAAT enhancer binding protein (C/EBP) can be eluted from DNA affinity columns by using heparin. Elution results from competition between the anionic polysaccharides (heparin and DNA) for transcription factor binding [9] and represents a novel elution method.

Lac repressor protein, which regulates the lac operon in *Escherichia coli,* has been studied extensively. A DNA operator sequence (Op1) which is bound by this protein has been identified [10]. We have shown previously [11] that lac repressor binds more tightly to columns having Op1 with an additional $(dA)_{18}$:$(dT)_{18}$ tail than to columns having Op1 alone. Two other transcription factors have also been investigated. C/EBP binds to CAAT elements in some eukaryotic and viral promoters and regulates gene expression [12–14]. B3 is a developmentally regulated transcription factor, which regulates TFIIIA transcription in early oocytes [15].

In this paper we describe a new method which we call the bi-column method for purification of transcription factors. This method involves the use of two columns having high specificity but different affinities for the transcription factor of interest. Proteins such as lac repressor-β-galoctosidase fusion protein (lacIZ), are eluted with heparin from a column having high specificity but moderate affinity (Op1-Sepharose). The eluate is passed over a second column, connected in tandem, having both high specificity and higher affinity. The proteins that are bound by the second column are finally eluted with salt. While we developed this method originally as a facile way to remove heparin from samples, the method has advantages for purification we did not anticipate. We have shown that this approach gives highly pure lac repressor, C/EBP and B3 proteins.

2. Methods

2.1. DNA Sepharose preparation

The following oligonucleotides were used:

Name	Sequence of strand which was coupled	Sequence of complementary strand
Op1	5′-NH_2-AATTGTTATCCGC-TCACAATTCCAC	5′GTGGAATTGTGAGC-GGATAACAATT
$Op1T_{18}$	5′-NH_2-$(T)_{18}$-AATTGTTAT-CCGCTCACAATTCCAC	5′GTGGAATTGTGAGCG-GATAACAATTA_{18}
$(EP18)_5$	5′-NH_2-$(GCAGATTGCGC-AATCTGC)_5$	NA
$(EP9)_{10}$	5′-NH_2-$(GCAGATTGC)_{10}$	$(5'GCAATCTGC)_{10}$
E3	5′-NH_2-TGTGGTTACTAGGTTAC-AAATTACCCTAGCAACCATG	5′-CATGGTTGCTAGGGTAAT-TTGTAACCTAGTAACCACA
E3/2	5′-NH_2-AAATTACCCTAGCAAC-CATGCATT	5′-AATGCATGGTTGCTAGGG-TAATTT

"5′-NH_2" represents an aminoethyl group added on the last synthesis cycle with the Aminolink reagent (Applied Biosystems). All strands having Aminolink were coupled to CNBr-preactivated Sepharose 4B (Sigma, St. Louis, MO, USA). Coupling and end-capping were carried out according to the protocol provided by the manufacturer. The columns were made double stranded by adding the corresponding complementary strand. The mixture was then heated to 95°C and allowed to cool slowly to room temperature. $(EP18)_5$ is self complementary and does not require the addition of a complementary strand. The amount of DNA coupled was determined by the difference in UV absorption of DNA added and recovered after coupling. Amounts of 115 μg of $(EP18)_5$, 95 μg of $(EP9)_{10}$, 177 μg of Op1, 160 μg of $Op1T_{18}$, 230 μg of E3/2 and 253 μg of E3 were found to be coupled/g Sepharose.

2.2. Production of proteins

Lac repressor-β-galactosidase (lacIZ) fusion protein was produced as described earlier [9] by growing clone BMH-72-19-1 which was generous gift of Dr. David Levens (Laboratory of Pathology, National Cancer Institute, Bethesda, MD, USA).

TB1 cell extract was obtained by using the same protocol used for production of lacIZ the only difference was that *E. coli* strain TB1 containing plasmid pMalC (New England Biolabs, Beverly, MA, USA) was used in place of clone BMH-72-19-1.

Rat liver nuclear extract used for studies on C/EBP was prepared by the procedure described in Ref. [16].

Xenopus laevis oocyte extract used for B3 purification was made from stage I–II *Xenopus* oocytes as described in Ref. [15].

2.3. Chromatography

All supports were packed in 1-ml bed volume syringe columns initially equilibrated in TE0.1 buffer (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 0.1 *M* NaCl). Details of elution and the gradient used are given in the figure legends.

2.4. Assay of lacIZ

LacIZ was assayed for β-galactosidase activity by using the protocol described previously [9].

2.5. Polyacrylamide gel electrophoresis

All samples were concentrated using Centriplus centrifugal filter devices supplied by Millipore (Bedford, MA, USA). One fourth of each sample was applied to sodium dodecylsulfate–polyacrylamide 12% gels using the method of Laemmli [17] and stained with silver using the Bio-Rad kit (Richmond, CA, USA).

2.6. Western blot analysis

Electrophoresis was carried out as above, proteins were then transferred to nitrocellulose filters and probed with appropriate antibody as described by Towbin et al. [18]. For lac repressor, 1:5000 diluted rabbit anti-lac repressor polyserum (Stratagene, La Jolla, CA, USA) was used as primary antibody. For C/EBP 1:5000 dilution of anti-C/EBP (14AA) supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA) was used as primary antibody. A 1:5000 dilution of rabbit polyserum generated against purified B3 (HTI Bioproducts) was used as primary antibody for detection of B3. Immunoreactive proteins were visualized by using 1:3000 diluted rabbit secondary antibody alkaline phosphatase conjugates and stained for alkaline phosphatase using a kit supplied by Promega (Madison, WI, USA).

3. Results and discussion

Fig. 1 shows elution of lac repressor-β-galactosidase (lacIZ) fusion protein from Op1 and $Op1T_{18}$ columns. It can be seen that lacIZ elutes from Op1 as a sharp peak at heparin concentration from 10 to 20 mg/ml with the peak fraction at 16 mg/ml. The peak of lacIZ eluted from $Op1T_{18}$ is fairly broad, with lacIZ eluting at heparin concentrations as low as 10 mg/ml, however, the bulk of lacIZ elutes at heparin concentrations between 25 and 40 mg/ml with the peak fraction at 32 mg/ml. These data are in agreement with our previous report where we had shown a similar difference in the salt elution of this protein from these two columns [11]. The $Op1T_{18}$ column shows some heterogeneity in eluted peak. We have found that some proteolysis occurs in the lacIZ protein. Since both β-galactosidase and lac repressor form tetramers a large number of species could be formed with truncated forms of lacIZ and this probably accounts for the heterogeneity observed.

Fig. 2 shows a diagrammatic presentation of the bi-column method. Column A in the figure represents a column such as Op1-Sepharose, which has a moderate affinity for the protein to be purified. Because of the moderate affinity, proteins elute from this column at lower heparin concentration. Column B represents columns such as $Op1T_{18,}$ which have a greater affinity for the protein. Although column A has a moderate affinity, it is by no means a non-specific column as it includes either the entire 25 base pair Op1 sequence bound by lac repressor protein or a part of a known DNAse1 footprint (EP9 or E3/2) bound by C/EBP and B3, respectively.

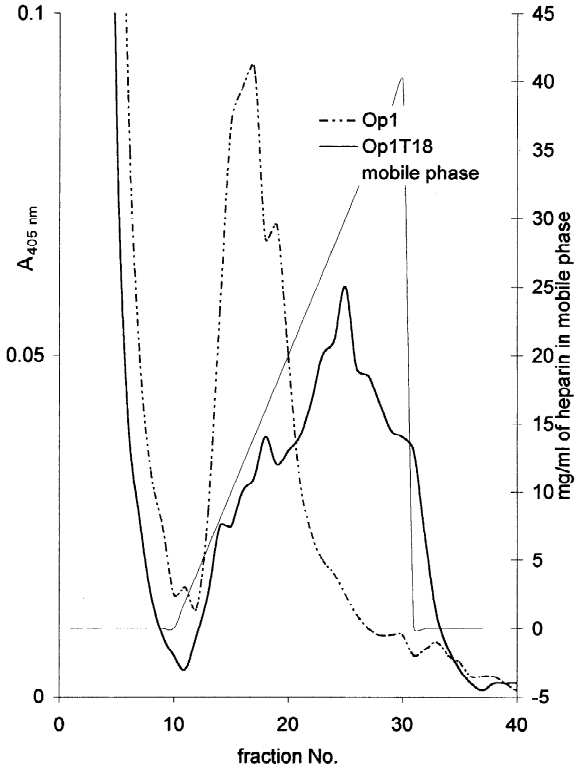

Fig. 1. Elution of lacIZ from Op1-Sepharose and $Op1T_{18}$-Sepharose with heparin. A 100-μl volume of crude bacterial extract containing lacIZ was loaded onto either Op1-Sepharose or $Op1T_{18}$-Sepharose, the columns were washed with 10 ml of TE0.1 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 0.1 *M* NaCl) and eluted with a 20-ml gradient of heparin from 0 to 40 mg/ml in TE0.1 followed by one more wash with 10 ml TE0.1. The flow-rate was 0.5 ml/min and 1-ml fractions were collected.

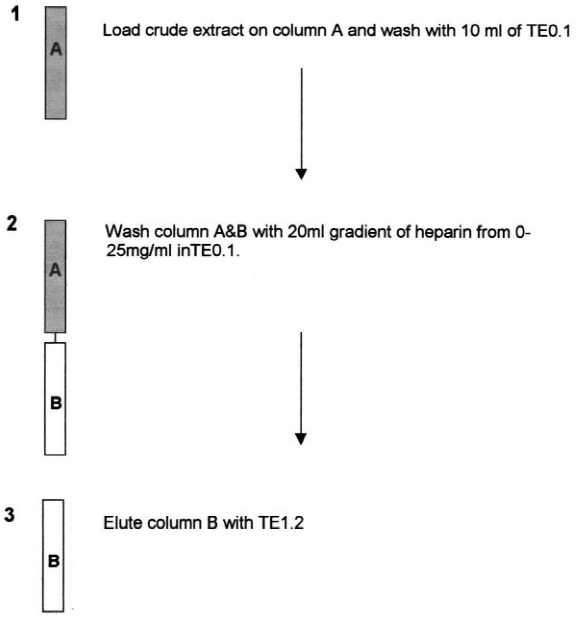

Fig. 2. Diagrammatic representation of bi-column method. Column A represents Op1, $(EP9)_{10}$ or E3/2-Sepharose. Column B represents $Op1T_{18}$, $(EP18)_5$ or E3-Sepharose. TE1.2 is 10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 1.2 *M* NaCl.

Column B on the other hand has the entire footprint region (EP18 and E3) and sometimes a footprint with additional sequences attached to it ($Op1T_{18}$) which increases binding affinity. Crude protein extract is loaded onto column A which is washed with TE0.1. Column B is then attached downstream of column A. A heparin gradient is applied to the bi-column at concentrations which will elute the protein from column A but not from column B. Column B is then separated and eluted with buffer TE1.2 which contains 1.2 *M* NaCl to obtain the purified transcription factor free of heparin.

Fig. 3 shows such a bi-column experiment for lacIZ. Op1-Sepharose and $Op1T_{18}$-Sepharose are used as columns A and B, respectively. Most of the lacIZ that is eluted from Op1 with heparin in step 2 is able to bind to the $Op1T_{18}$ column and very little passes through the column as can be seen in fractions 20–35 in Fig. 3. LacIZ then elutes as a sharp peak in fractions 40–45 from the $Op1T_{18}$-Sepharose column with TE1.2.

The bi-column method also works with the $lacI^q$ mutant of repressor protein expressed from low copy number pMalc plasmid. It can be seen from Fig. 4 that highly pure lac repressor protein is obtained with the bi-column method (lane 1) when a crude bacterial extract is used as the starting material. It can also be seen from the figure that protein obtained from the same extract with salt elution alone of $Op1T_{18}$-Sepharose is highly impure and several bands of protein impurities can be seen on the gel (lane 2). Lanes 3 and 4 show a Western blot of protein purified with the bi-column and single column salt elution, respectively.

Fig. 5 shows a bi-column purification of C/EBP from rat liver nuclear extract. $(EP9)_{10}$-Sepharose which has only half of the binding site for C/EBP was used as column A and $(EP18)_5$-Sepharose which

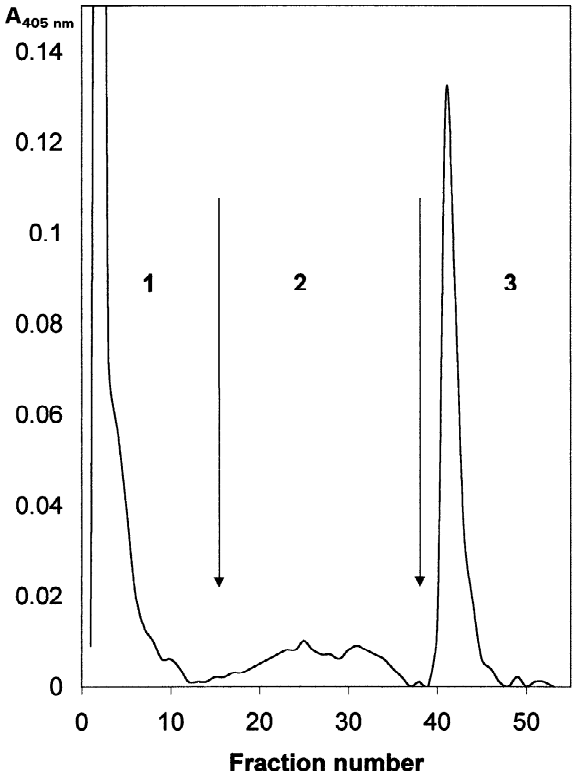

Fig. 3. Bi-column elution of lacIZ. A 300-μl volume of crude extract containing lacIZ was loaded onto Op1-Sepharose and the bi-column elution was carried out as described in Fig. 2. Op1-Sepharose was used as column A and $Op1T_{18}$-Sepharose was used as column B. The flow-rate was 0.5 ml/min and 1-ml fractions were collected.

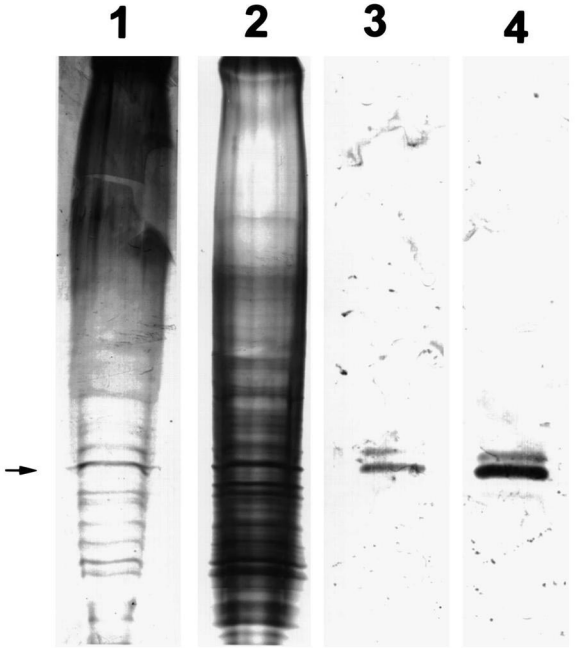

Fig. 4. The bi-column method works better for purification of lac repressor than salt elution. A 500-μl volume of crude extract containing $lacI^q$ produced from a low copy number plasmid was loaded onto Op1-Sepharose and the bi-column elution was carried out as in Fig. 2 except a 10-ml wash with TE0.4 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 0.4 *M* NaCl) was applied to column B ($Op1T_{18}$-Sepharose) before eluting the protein with TE1.2. For salt elution, 500 μl of crude extract containing lac repressor was loaded onto $Op1T_{18}$-Sepharose, the column was washed with 30 ml TE0.1 followed by a 10-ml wash with TE0.4, and eluted with 10 ml TE1.2. Lanes 1 and 2 are from a silver stained gel while lanes 4 and 5 are Western blots of bi-column purified and salt eluted lac repressor, respectively.

consists of five tandem copies of the entire DNA binding sequence was used as column B. We found that Green fluorescent protein-CAAT enhancer binding protein chimeric fusion protein (GFP-C/EBP) binds to $(EP9)_{10}$ but elutes at a lower heparin concentration than from $(EP18)_5$ (data not shown), hence these two columns were used. It can be seen from the figure that the bi-column method gives highly pure protein and only two or three contaminant protein bands can be seen after silver staining (lane 1). Protein purified with single step salt elution on the other hand looks very impure and several other contaminant protein bands can be seen along with that of C/EBP on the gel (lane2). Lanes 3 and 4 show Western blots of the samples used for lanes 1 and 2. Notice that so little C/EBP elutes with salt that it can be barely detected with antibody while with the bi-column method, it is a major constituent.

Transcription factor B3 regulates TFIIIA transcription in early *Xenopus* oocytes. The DNA binding region of B3 consists of four dyads. It has been reported earlier [15] that B3 binds with a lower affinity to a sequence containing dyads 3 and 4 (E3/2) than to sequence containing all four dyads (E3). Hence we used E3/2-Sepharose and E3-Sepharose as column A and column B in the bi-column method, respectively. It can be seen from Fig. 6 that the bi-column method works very well for purification of B3 from *Xenopus* oocyte extract and a single band of B3 can be seen after silver staining (lane 1).

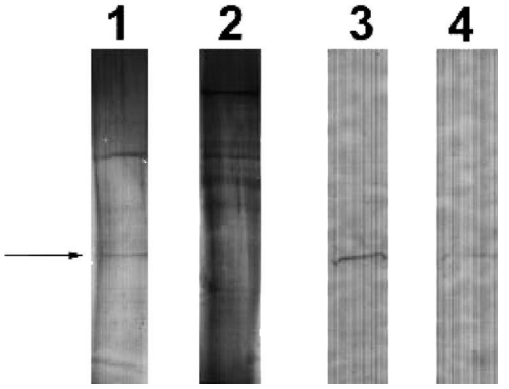

Fig. 5. Bi-column method and salt elution purification of C/EBP. A 500-μl volume of rat liver nuclear extract containing C/EBP was loaded onto $(EP9)_{10}$-Sepharose and the bi-column experiment was carried out as described in Fig. 2. $(EP9)_{10}$- and $(EP18)_5$-Sepharose were used as columns A and B, respectively. A 10-ml wash with TE0.4 was applied to column B before eluting the protein with TE1.2. For salt elution, 500 μl of rat liver nuclear extract containing C/EBP was loaded onto $(EP18)_5$-Sepharose. The column was washed with 30 ml TE0.1, followed by a 10-ml wash with TE0.4, and eluted with 10 ml TE1.2. Lanes 1 and 2 represent a silver stained gel while lanes 3 and 4 are Western blots of C/EBP purified with bi-column and salt elution, respectively.

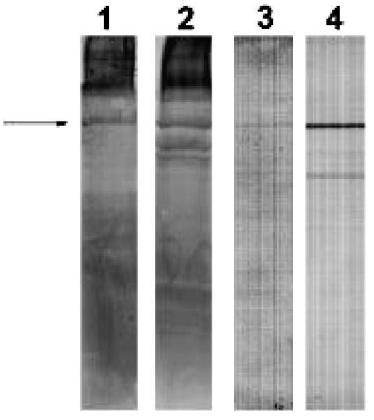

Fig. 6. Bi-column and salt elution for purification of B3. 500 μl of *Xenopus* oocyte extract containing B3 transcription factor was loaded onto E3/2-Sepharose and the bi-column experiment was carried out as depicted in Fig. 2. E3/2-Sepharose and E3-Sepharose were used as columns A and B, respectively. A 10-ml wash with TE0.4 was applied to column B E3-Sepharose) before eluting the protein with TE1.2. For salt elution, 500 μl of *Xenopus* oocyte extract containing B3 was loaded onto E3-Sepharose, the column was washed with 30 ml TE0.1, followed by a 10-ml wash with TE0.4, and eluted with 10 ml TE1.2. Lanes 1 and 2 represent silver stained gel while lanes 3 and 4 are Western blots of B3 purified with the bi-column and salt elution, respectively.

As for C/EBP and lac repressor, conventional salt elution gives a highly impure protein and several other protein bands are visible on the gel (lane 2). The Western blot however confirms that salt elution gives higher yield.

We have shown earlier that lacIZ eluted with heparin from a $Op1T_6$ columns is highly pure [9]. Although heparin eluted protein is highly pure, heparin has to be removed later since it can interfere with the DNA binding activity of the protein. The bi-column method was initially developed to remove heparin. We found out that the bi-column methods not only performs this function but is also very effective way of purifying transcription factors.

Highly pure lac repressor, C/EBP and B3 can be obtained, from their respective sources, bacterial extract, rat liver nuclear extract and *Xenopus* oocyte extract by using the bi-column method alone. The bi-column method is probably effective because it combines the use of two different specific DNA affinity columns and two different elution methods. In the bi-column method the proteins shuttles from DNA on column A to heparin in mobile phase and back to DNA on column B. The protein to be purified has a higher affinity for column B and hence can be retained by it. Proteins, which are nonspecifically bound to column A on the other hand probably, have similar affinities for both columns and hence are not retained by column B.

4. Conclusions

The bi-column method could be extended to purify any transcription factor as long as its DNA element has been, or can be, identified and two high specificity columns having different affinities for the transcription factor can be generated.

This bi-column protocol yields higher purity of B3 that has ever been reported, even using much more complex purification schemes involving several different chromatographic steps. The C/EBP obtained is also of a higher purity than has ever been obtained from such a simple technique as the bi-column [19]. These purifications are all from less than 1 ml of a crude extract containing only native or near native amounts and yet could be purified to levels which would be sufficient for characterization.

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