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Molecular Cloning of the Complementary DNA for a Human Folate Binding Protein[1] (42804)

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Abstract. The complementary DNA for a human folate binding protein has been cloned from a λgt11-cDNA library prepared from cultured KB cells. A number of clones were selected by immunoscreening with a monospecific antiserum and by oligonucleotide probes corresponding to the NH_2-terminal sequence of the folate binding protein. A partial nucleotide sequence of the cDNA was determined directly from the λgt11 phage and after subcloning into M13. The 18 amino acids deduced from the initial 19 codons were exactly the same as the amino acid sequence obtained by peptide analysis of the purified protein providing proof that this clone is the folate binding protein cDNA.

Procaryotic and eucaryotic cells express proteins which bind folic acid and some reduced folate derivatives (1). These proteins, generically named folate binding proteins (FBP),[4] have been isolated from cell membranes (2–5) and cytosol and secretions (6–8).

The KB cells, a cultured cell line derived from a human epidermoid carcinoma, have provided an opportunity to study the structural and functional properties of the FBPs because these cells express both a membrane-associated hydrophobic and a hydrophilic-secreted form of this protein (7). This cell line, therefore, provides a good model to characterize the functional–structural elements of this protein. Toward this end, we have prepared a complementary DNA (cDNA) library from KB cells from which we isolated the FBP cDNA by screening with an antiserum to the FBP and an oligonucleotide probe corresponding to an amino acid sequence of the protein (9). The exact concordance of the deduced amino acid sequence of the cDNA corresponded exactly with the amino acid sequence derived by peptide analysis of the purified protein providing proof that we have isolated the FBP cDNA clone.

Materials and Methods. *Construction of the λgt11 cDNA library.* Total RNA was prepared from cultured KB cells by the method of Chirgwin *et al.* (10). The poly(A)$^+$ RNA was isolated by chromatography twice through the oligo(dt)-cellulose column. Synthesis of the cDNA, the *Eco*RI methylation, *Eco*RI linker ligation, and the *Eco*RI digestion followed the method of Okayama and Berg (11) as modified by Gubler and Hoffman (12). After the linked cDNA was ligated to the λgt11 arms (13), it was packaged following the instructions provided with the packaging kit from Stragagene (San Diego, CA). *Escherichia coli* (strain 1090) was then infected by the phage particles and cultured on LB agar plates. The library had a titer of 3.9×10^5 recombinant plaques/ml.

Screening of the cDNA library. The cDNA library was screened for the FBP cDNA using an oligonucleotide probe and for expression of the FBP by immunostaining with an antiserum raised to the purified protein from KB cells (7).

A mixture of oligonucleotides (18 mer) corresponding to the 13 to 18 amino acids (Met–Asn–Ala–Lys–His–His) of the NH_2-terminal domain of the protein was synthesized by Dr. William McAllister (Department of Immunology and Microbiology,

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[4] Abbreviations used: FBP, folate binding protein; IPTG, isopropyl β-D-thiogalactopyranoside; cDNA, complementary DNA.

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SUNY Health Science Center, Brooklyn, NY).[5] The mixture of oligonucleotides (1 μg) was end-labeled with [γ-^{32}P]ATP and hybridized in 6× SSC (sodium chloride, 0.15 *M*; sodium citrate, 0.015 *M*) at 42°C to the plaques transferred to the nitrocellulose filters (14). The filters were washed several times in 6× SSC at 42°C with a final wash for 5 min at 47°C in the same buffer. The filters were air-dried and exposed for 16–72 hr to Cronex 7 X-ray film at −70°C using an intensifying screen. A total of six positive cDNA clones were selected and plaque purified.

To determine which of these six clones also expressed protein which cross-reacted with the antiserum to the purified FBP, the infected *E. coli* were plated in LB agarose at the density of 200 plaques/plate at 42°C for 3.5 hr. The plates were then overlaid with IPTG-treated cellulose nitrate filter paper and incubated at 37°C for another 3.5 hr to induce the synthesis of the fusion protein. The filter was then treated sequentially with a dilution of rabbit antiserum (0.015 A_{280} units/ml) raised to the purified KB FBP and which was purified by affinity chromatography using protein-A coupled to Sepharose (15). The filter was then processed for immunostaining with goat antiserum to rabbit γ-globulin conjugated to alkaline phosphatase and the chromogen developer, nitroblue tetrazolium, and the substrate, 5-bromo-4-chloro-3 indolyl-phosphate, using the Proto Blot immuno-screening system instructions provided by Promega (Madison, WI).

Four of the six clones selected by hybridization with the oligonucleotide probe also expressed the immunoreactive protein. One of the clones which expressed the fusion protein was subjected to DNA sequencing by the dideoxy method of Sanger *et al.* (16).

DNA sequencing. The important first objective in determining the nucleotide sequence of the cDNA was to obtain proof that it was the FBP cDNA clone. This can be definitively established by showing that the deduced amino acid sequence is the same as the amino acid sequence determined by peptide analysis of the protein.

We first sequenced the cDNA directly from the insert in the λgt11 phage because of the apparent convenience of this method. The phages were purified from the bacterial lysate using Lambda Sorb supplied by Promega (Madison, WI). The DNA was extracted from the phage as described by the manufacturer and denautred by alkali and then annealed to sequencing primers purchased from New England Biolabs (Beverly, MA) which were first end-labeled with [^{32}P]ATP. Approximately 2 μg of the alkali denatured viral DNA was annealed to the primers and the sequencing protocol followed was provided by the manufacturer (New England Biolabs). Each of the four sequencing reactions (3 μl) was loaded into a 6% polyacrylamide gel containing 8 *M* urea and subjected to electrophoresis at 50 W until the bromphenol blue tracking dye reached the bottom of the gel. The gels were then dried and exposed to Kodak XAR-2 film for 16–72 hr at −70°C.

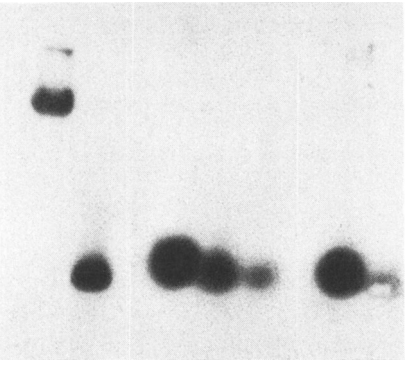

FIG. 1. Southern Blot of DNA from clones 1–6. The purified DNA was digested with *Eco*RI and electrophoresed in a 1.5% agarose gel. After denaturation with alkali, the DNA was transferred to Zeta Probe membranes (Bio-Rad Laboratories, Richmond, CA) under conditions provided by the manufacturer. The blot was then hybridized in the ^{32}P end-labeled oligonucleotide mixture as described under Methods and exposed to Cronex-7 X-ray film for 5 min at room temperature. Lanes 2–7: *Eco*RI digest of DNA from clone 6, 1, 2, 3, 4, and 5, respectively. Lane 1: Undigested DNA from clone 6.

[5] The oligonucleotide probes were prepared using a Biosearch 8600 automated DNA synthesizer using β-cyanoethyl protected phosphoramidite chemistry. The products were cleaved by base and purified after deprotection by reverse phase HPLC using a Beckman C-3 column.

The cDNA was also sequenced using the M13 subclone in order to clarify the initial few nucleotides in the 5′ terminus which were unclear using the λgt11 clone. The cDNA was inserted into *Eco*RI restriction sites of M13 and sequenced using Sequenase and the M13 universal primer (U.S. Biochemicals Corp.).

Results. Figure 1 shows a Southern blot of the *Eco*RI digest of the cDNA clones probed with the ^{32}P-labeled oligonucleotide preparation. The digested DNA from clone 6 is shown in lane 2 and the digests of clones 1–5 are shown in lanes 3 to 7, respectively. The probe hybridized with a 1000-bp insert in each of the DNA digests. The Southern blot of DNA from clone 6, which was not digested with *Eco*RI, is shown in lane 1. In this instance, the oligonucleotide probe hybridized with the high-molecular-weight phage DNA which contains the insert sequence, providing additional evidence for the specificity of hybridization to the cloned cDNA. In addition, this clone was plated with IPTG, and it expressed a fusion protein which reacted with a rabbit antiserum raised to the purified FBP from KB cells. The partial nucleotide sequence of clone 5 is shown in Table I.

Table II shows the deduced amino acid sequence of the cDNA clone and its homology to the published NH_2-terminal sequences of the KB FBP (9), the human milk FBP (17), and the bovine milk FBP (18). The first amino acid derived from the nucleotide sequence is threonine (assigned position 4) and this actually precedes the published NH_2-terminal end residue, isoleucine, for both the KB and human milk FBPs. However, the arginine residue (following the threonine) is identical to the published residue 5 of the bovine milk FBP (18). The amino acids at assigned residues 24 and 25 in the clone are lysine and glutamic acid, respectively, which are identical to residues 19 and 20 of human milk FBP.

Discussion. In a previous preliminary report[6] we demonstrated that the cDNA library prepared from KB cell messenger RNA (mRNA) contained one clone which ex-

TABLE I. PARTIAL NUCLEOTIDE SEQUENCE OF THE CODING REGION OF THE HUMAN FOLATE BINDING PROTEIN cDNA AND THE DEDUCED AMINO ACID SEQUENCE

Nucleotide number	10	20	30	40	50	60	70	75
	GAA TTC CGG ACA AGG ATT GCA TGG GCC AGG ACT GAG CTT CTC AAT GTC TGC ATG AAC GCC AAG CAC CAC AAG GAA							
	Thr Arg Ile Ala Trp Ala Arg Thr Glu Leu Leu Asn Val Cys Met Asn Ala Lys His His Lys Glu							

[6] Part of this work was presented at the 1987 National Meeting of the Federation of American Societies for Experimental Biology.

TABLE II. NH_2-TERMINAL AMINO ACID SEQUENCES OF THE FBPS

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	—	—	—	Thr	Arg	Ile	Ala	Trp	Ala	Arg	Thr	Glu	Leu
2	—	—	—	—	—	Ile	Ala	Trp	Ala	Arg	Thr	Glu	Leu
3	—	—	—	—	—	Ile	Ala	Trp	Ala	Arg	Thr	Glu	Leu
4	Ala	Gln	Ala	Pro	Arg	Thr	Pro	Arg	Ala	Arg	Thr	Asp	Leu
	14	15	16	17	18	19	20	21	22	23	24	25	
1	Leu	Asn	Val	Cys	Met	Asn	Ala	Lys	His	His	Lys	Glu	
2	Leu	Asn	Val	Cys	Met	Asn	Ala	Lys	His	His	—	—	
3	Leu	Asn	Val	—	Met	Asn	Ala	Lys	His	His	Lys	Glu	
4	Leu	Asn	Val	Cys	Met	Asp	Ala	Lys	His	His	Lys	Ala	

Note. Sequence 1 was deduced from the cDNA clone; sequence 2 is the published NH_2-terminal sequence of the KB FBP; sequence 3 is the published NH_2-terminal sequence of the human milk FBP; sequence 4 is the published NH_2-terminal sequence of the bovine milk FBP.

pressed a cross-reacting peptide with the FBP. We have now screened the same library with oligonucleotide probes corresponding to amino acids 13 to 18 of the amino terminus of the protein and identified six clones which contain very similar size cDNA inserts of approximately 1000 bp and each insert hybridized with the oligonucleotide screening probe by Southern blot analysis (Fig. 1). It is not clear why the cDNA inserts of these six clones were of such similar size. It is possible that this was an artifact of the amplication of the cDNA library and the clones containing this 1000-bp insert grew more efficiently and were then selected on a random basis by the oligonucleotide probe in the screening procedure (12). The similar size of each insert may explain why four of the six clones (rather than the expected two of six clones) expressed an immunoreactive protein, in that the nucleotide sequence of the reading frame of each cDNA clone was in phase with the β-galactosidase reading frame sequence. The nucleotide sequence of clone 5, in fact, shows that it is in phase with the β-galactosidase reading frame. With proper phasing between the β-galactosidase coding region and the cDNA insert, the chance of expressing a fusion protein is three of six clones depending on the 50/50 chance that the cDNA was inserted in the phage in the proper orientation. The nucleotide sequence of clone 4 (not shown), which did not express an immunoreactive fusion protein, showed that it was ligated into the 5′ end of the β-galactosidase sequence by its poly(A) end.

The fact that the deduced amino acid sequence of the open reading frame of clone 6 corresponds exactly to the amino acid sequence of this amino terminal region of the FBP proteins confirms that this clone contains the FBP cDNA. Moreover, since the cDNA was synthesized from the 3′ poly(A) region of the mRNA in the 5′ direction, it is likely to contain the full-length coding sequences of the FBP gene. The sequence identity of the KB FBP with the human milk FBP and the homology observed with the FBP from boving milk provides additional evidence that this clone contains the FBP cDNA.

Although clone 6 contains the FBP cDNA, it is lacking the methionine residue and the leader peptide required for translocation and processing the translated protein through the endoplasmic reticulum (19). To characterize this region of the FBP gene we are screening other clones from the original library to find larger cDNA inserts as well as a genomic library prepared from human fetal liver.

It should be noted that although isoleucine is the initial NH_2-terminal amino acid when the purified FBP from KB cells was directly sequenced (9), threonine is the initial amino acid deduced from cDNA clone. Isoleucine is also reported to be in the NH_2-terminus of the human milk folate finding protein by Svendsen *et al.* (17). Thus the KB and human milk FBPs appear to be shorter by five amino acid residues than the bovine milk FBP. This finding could be explained in two ways. Because arginine in the deduced

position preceding isoleucine in the KB FBP is in the analogous position to arginine in the bovine milk FBP, it is possible that there are actually four additional residues preceding the isoleucine in the KB and human milk FBP which were lost as a consequence of proteolysis during the purification of the proteins, as suggested by Svendsen *et al.* (17). A second possibility is that the isoleucine is the correct NH_2 terminus of the human FBPs and the deduced threonine and arginine residues in the KB FBP are part of the signal peptide which is removed in the cellular processing of the protein. Additional studies on the gene isolated from the genomic library will clarify this question.

This is the first report of the molecular cloning of the cDNA for a human FBP. A recent study by Antony and co-workers (20), which showed that antiserum to a human FBP blocked folate uptake and impaired the normal maturation of human erythroid colonies, provides important evidence for the role of this protein in the cellular uptake of folate. The isolation of the cDNA for this FBP will assist in establishing the sites of glycosolation and acylation and the integration of the hydrophobic domain(s) into the cell membrane. This could help elucidate how the protein facilitates the cellular uptake of folate and should give us an opportunity to clarify a number of questions about the biologic properties of this protein.

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