Molecular Cloning and Characterization of Recombinant Parasite Antigens for Immunodiagnosis of Onchocerciasis

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Abstract

Immunological cross-reactivity among nematodes has hampered the development of specific serodiagnostic assays for onchocerciasis. In the present study, an *Onchocerca volvulus* adult worm complementary DNA expression library was differentially screened with human sera from patients infected with *O. volvulus* and with an omnibus anti-nematode serum pool comprised of sera from patients infected with *Brugia malayi, Loa loa, Wuchereria bancrofti, Mansonella perstans, Strongyloides stercoralis, Ancylostoma duodenale, Ascaris lumbricoides,* and *Dracunculus medinensis.* Seven *Onchocerca*-specific clones were identified and screened with individual onchocerciasis patient sera. Additional studies were performed to characterize the most immunoreactive clones, OC 3.6 and OC 9.3. OC 3.6 produced a 152-kD β-galactosidase fusion protein that was recognized in dot-immunoblots by 54 of 55 sera from onchocerciasis patients (98%). The OC 3.6 DNA insert is 996 bp long with an open reading frame of 627 bp and a 369-bp untranslated 3′ end. OC 3.6 is closely related to a previously reported clone (OV 33-3), but it differs from that clone at both the 5′ and 3′ ends. OC 9.3 contained a novel 565-bp insert and produced a 138-kD fusion protein that was recognized by 46 of 55 sera from onchocerciasis patients (83%). Additional studies are in progress to develop and evaluate immunodiagnostic tests for onchocerciasis based on measurement of antibodies to these promising recombinant antigens. (*J. Clin. Invest.* 1991. 88:1460–1466.) Key words: filariasis • nematode • *Onchocerca*

Introduction

Onchocerciasis, a disease caused by the filarial nematode *Onchocerca volvulus,* affects an estimated 20 million people in Africa and Latin America (1). The parasite is transmitted by bites of *Simulium* black flies. The most important clinical manifestations of onchocerciasis are blindness and severe chronic dermatitis caused by immunopathological reactions to parasite larvae (microfilariae) in tissues (1). At present, the parasitological diagnosis of human onchocerciasis depends primarily on the demonstration of microfilariae in skin snips. However, skin snip examination is not sensitive for detection of early infections or for diagnosis of individuals with low microfilaria densities in skin (2, 3). Improved methods for diagnosis of such infections would aid in the assessment of control programs that attempt to interrupt transmission of onchocerciasis by vector control (4) or by mass chemotherapy with the microfilaricide ivermectin (5).

Attempts to develop antibody serological tests for early diagnosis of onchocerciasis have been hampered by poor specificity caused by immunological crossreactivity between *O. volvulus* and sympatric parasites. To be useful, an immunodiagnostic test for onchocerciasis should be at least as sensitive as skin snip examination, provide a species-specific diagnosis, and be practical for use in endemic areas. In addition to antigenic sharing, a second problem that has inhibited progress is that *O. volvulus* parasite material is very difficult to obtain; the worms cannot be propagated in vitro or in laboratory animals and must be manually isolated from subcutaneous nodules that are surgically removed from human patients. To some extent, such problems can be side-stepped by application of molecular biological methods that allow efficient screening of DNA expression libraries to identify potentially useful recombinant antigens and production of selected antigens in quantities without requiring additional parasite material. This article describes studies performed to clone and characterize recombinant *O. volvulus* antigens that might be useful for immunodiagnosis of onchocerciasis.

Methods

Human sera. Nigerian sera were collected in Jago, a forest zone village near Ibadan, Nigeria. The diagnosis of onchocerciasis was based on clinical examination for subcutaneous nodules or onchocercal dermatitis and examination of at least four skin snips per person as previously described (6). Guatemalan onchocerciasis sera were collected from residents of coffee plantations in the Lake Atitlan endemic area (municipalities of Acatenango and Chicacao). Residents of villages that are endemic for onchocerciasis who had negative skin snip examinations and negative clinical evaluations were classified as endemic controls. Nonendemic control sera were obtained from adult residents of Ibadan, Nigeria, and Guatemala City who had no history of residence in onchocerciasis endemic areas and from healthy residents of St. Louis, MO. Additional control sera were from patients with parasitologically documented *Strongyloides stercoralis, Ascaris lumbricoides, Ancylostoma duodenale, Wuchereria bancrofti, Brugia malayi, Loa loa, Dracunculus medinensis, Mansonella perstans,* or *M. ozzardi* infections. Some of the *L. loa* and *Mansonella* serum samples were provided by the World Health Organization Filariasis Serum Bank (Dr. N. Weiss, Swiss Tropical Institute, Basel). The *L. loa* and *M. perstans* serum samples were collected from persons who reside in Africa and who may have been exposed to *O. volvulus.* All of the other sera were collected from areas that are nonendemic for onchocerciasis.

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Table I. Immunoreactivity of Recombinant O. volvulus Antigens with Human Serum Pools and Rabbit Immune Sera*

Clone No.	Immunoreactivity: Onchocerciasis: Children	Immunoreactivity: Onchocerciasis: Adults	Immunoreactivity: Rabbit anti-*O. volvulus*	DNA insert‡
				kb
OA 4.2	+	+	−	0.6
OA 6.6	+	+	−	0.5
OC 1.4	+	+	−	0.6
OC 3.6	+	+	+	1.0
OC 6.3	+	−	−	0.4
OC 7.2	+	−	+	0.6
OC 9.3	+	+	+	0.6

* A + indicates strong reactivity by dot immunoblot; a − indicates no specific reactivity.
‡ DNA insert size determined by PCR.

An omnibus anti-nematode serum pool was prepared with sera from patients infected with *B. malayi, L. loa, W. bancrofti, M. perstans, S. stercoralis, A. duodenale, A. lumbricoides,* and *D. medinensis* for use in screening recombinant phage as described below.

Immunoscreening. A lambda gt11 cDNA *O. volvulus* adult worm gene expression library (7) was obtained from American Type Culture Collection, Rockville, MD (Kumba, catalogue no. 37509). Preliminary studies indicated that this amplified library had a titer of 10^6 plaque-forming units (pfu)/ml and produced 93% white plaques when it was plated onto a lawn of *E. coli* Y1090 with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)[1] indicator. The library was immunoscreened to identify *O. volvulus*-specific clones essentially as described by Young and Davis (8). Briefly, phage were plated onto a lawn of *E. coli* Y1090 at a density of 10,000 phage per petri dish (150 cm^2) and grown at 42°C for 3 h. When plaques were visible, isopropyl-β-D thiogalactoside (IPTG)-impregnated nitrocellulose filters were placed on the plates for 3 h at 37°C. Filters were removed and fresh filters were placed on plates for 3 h to obtain a second plaque lift. After blocking in 0.01 M phosphate-buffered saline, pH 7.4, with 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) (PBS/T), filters were incubated in diluted sera at 4°C overnight with gentle rocking. Human serum pools were diluted 1:500 in PBS/T and absorbed with *E. coli* antigens before use. Antibody reactivity with recombinant proteins was revealed by incubation of filters with alkaline phosphatase conjugated goat anti-human IgG antibodies (Promega Biotec, Madison, WI) for 3 h at 37°C and development with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT, Sigma Chemical Co.). Clones that were reactive with the *O. volvulus* serum pool but not with the omnibus antinematode serum pool were selected and purified by repeated cycles of immune selection.

Plaque-dot immunoblots. The reactivity of serum pools and of individual human sera to fusion proteins expressed by purified recombinant phage was studied by plaque-dot immunoblot analysis. Recombinant phage (10^3 pfu in 1 μl) was spotted in a prearranged grid onto an *E. coli* Y1090 lawn on Luria-Bertani agar with ampicillin. Plates were incubated at 42°C until just before complete lysis of phage spots occurred. Nitrocellulose filters were placed on plates overnight at 32°C. After blocking, filters were cut into 0.8-mm strips and probed with sera that had been preadsorbed by incubation with *E. coli* antigens bound to nitrocellulose. The dot blots were then processed as described above to detect antibodies bound to recombinant protein except that individual human sera were tested at a dilution of 1:100.

Polymerase chain reaction (PCR). PCR was employed to amplify the cDNA inserts of selected recombinant lambda gt11 clones with the Gene Amp DNA amplification kit (Perkin Elmer-Cetus, Norwalk, CT) according to the manufacturer's instructions. The amplified products of PCR were identified and insert sizes were determined by agarose gel electrophoresis.

Immunoblot analysis of recombinant fusion proteins. E. coli Y1090 was infected at high density with recombinant phage on a thin layer of agarose over LB agar to achieve confluent lysis, and synthesis of fusion proteins encoded by cDNA inserts was induced with IPTG-impregnated filters as described above. Top agarose containing bacterial lysate and fusion protein was then gently scrapped off and dissolved in SDS-PAGE sample buffer. SDS-PAGE was performed as described by Laemmli (9) at 135 V in 8% reducing gels. After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membrane (BA 83; Schleicher & Schuell, Inc., Keene, NH) as described by Towbin et al. (10). After transfer, nitrocellulose membranes were blocked in 5% nonfat dry milk in PBS/T for 3 h at 37°C. Membranes were then incubated in monoclonal antibody to β-galactosidase (Promega Biotec) or in human sera diluted in PBS/T overnight at 4°C. Membranes were washed in PBS/T and incubated with alkaline phosphatase conjugated goat anti-mouse IgG or anti-human IgG (Promega Biotec) for 3 h at 37°C. After washing, membranes were developed with BCIP/NBT.

DNA sequencing. Lambda gt11 DNA purified from selected clones was digested with EcoR1 and ligated into the EcoR1 site of pBluescript II SK- (Strategene Cloning Systems, La Jolla, CA) by standard methods (11). Competent XL1-Blue cells were transformed with the ligation mix and plated onto LB-ampicillin plates with 40 μl of 2% X-gal and 100 μl of 0.1 M IPTG. The presence of DNA inserts in white colonies was determined by agarose electrophoresis of DNA isolated by the rapid boiling method (12). Next, plasmid DNA was prepared from selected colonies by standard methods for sequencing. The dideoxynucleotide chain termination method (13) was used for double stranded DNA sequencing using the TaqTrack Sequencing System (Promega Biotec) with T3 and T7 pBluescript primers and with synthetic oligonucleotide primers.

O. volvulus antigen. O. volvulus adult worm crude worm extract and rabbit antibodies to this antigen were produced as previously described (6).

Table II. Immunoreactivity of Human Sera with Recombinant O. volvulus Antigens*

Serum source	No. of sera tested	No. of sera reactive with: OC 3.6	No. of sera reactive with: OC 9.3
Onchocerciasis (Nigeria)	31	30	23
Onchocerciasis (Guatemala)	24	24	23
Endemic controls (Nigeria)	12	3	1
Endemic controls (Guatemala)	4	2	2
Nonendemic (Nigeria)	8	0	0
Nonendemic (Guatemala)	Pool	0	0
Nonendemic (United States)	10	0	0

* Immunoreactivity was assessed by dot-immunoblot as described in Methods.

1. *Abbreviations used in this paper:* BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; IPTG, isopropyl-β-D thiogalactoside; PCR, polymerase chain reaction; PBS/T, 0.01 M PBS, pH 7.4, with 0.05% Tween 20; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

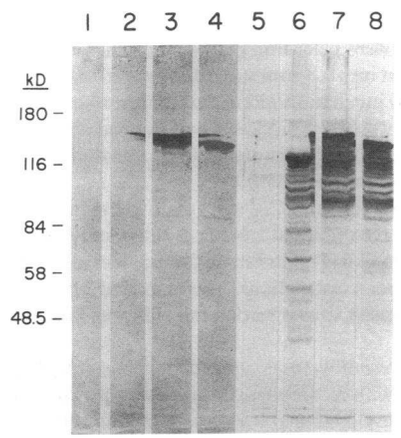

Figure 1. Western blot analysis of β-galactosidase/*O. volvulus* fusion proteins. *E. coli* Y1090 was infected with wild-type or recombinant λ gt11 and the synthesis of fusion proteins was induced by IPTG as described in Methods. Bacterial cell lysates were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose paper. Lanes *1* and *5* contain lysate from bacteria infected with wild-type λ gt11 (uninduced); lanes *2* and *6*, wild-type λ gt11 (IPTG induced); lanes *3* and *7*, recombinant phage OC 3.6 (induced); lanes *4* and *8*, recombinant phage OC 9.3 (induced). Lanes *1–4* were developed with a serum pool from humans with onchocerciasis. Lanes *5–8* were developed with a monoclonal antibody to β-galactosidase.

Computer analysis. The PC/GENE DNA Sequence Analysis System (IntelliGenectics, Inc./GENOFIT SA., Mountain View, CA) was used to analyze nucleotide and deduced peptide sequences. The Word Search Program (14) of the Genetics Computer Group (University of Wisconsin, Madison) sequence analysis package was used to search the GenBank and EMBL nucleic acid libraries. Homology of deduced amino acid sequences with previously reported sequences was determined with the FastP program (15) and the Protein Sequence Data Base of the Protein Identification Resource of the National Biomedical Research Foundation (Washington, DC).

Results

Selection and immunological characterization of λ gt11 clones that express O. volvulus-specific antigens. Approximately 400,000 phage plaques from an *O. volvulus* cDNA expression library were immunoscreened with the onchocerciasis serum pool and the omnibus anti–nematode serum pool as described in Methods. 19 clones selected in the initial screen were re-screened with separate serum pools from patients infected with each of the parasites represented in the omnibus serum pool. Seven *O. volvulus*-specific clones were identified. Immunoreactivity of these clones, as determined by plaque-dot blot analysis with human onchocerciasis serum pools and rabbit immune sera, is summarized in Table I along with their DNA insert sizes. All seven clones were recognized by a serum pool comprising 12 sera from infected children (8–15 yr of age) from Jago, Nigeria, but only five were recognized by a serum pool comprising 12 sera from infected adults from the same village. Interestingly, rabbit antibodies to *O. volvulus* reacted with only three of these clones. Immunoreactivity of individual human sera with these clones is summarized in Table II. The most immunoreactive clones were OC 3.6 and OC 9.3, which were recognized by 98% and 83% of onchocerciasis patient sera, respectively. Some of the endemic control sera also contained antibodies to antigens produced by these clones. These antibodies may indicate the presence of early infections in these individuals. Alternatively, antibody responses to the recombinant

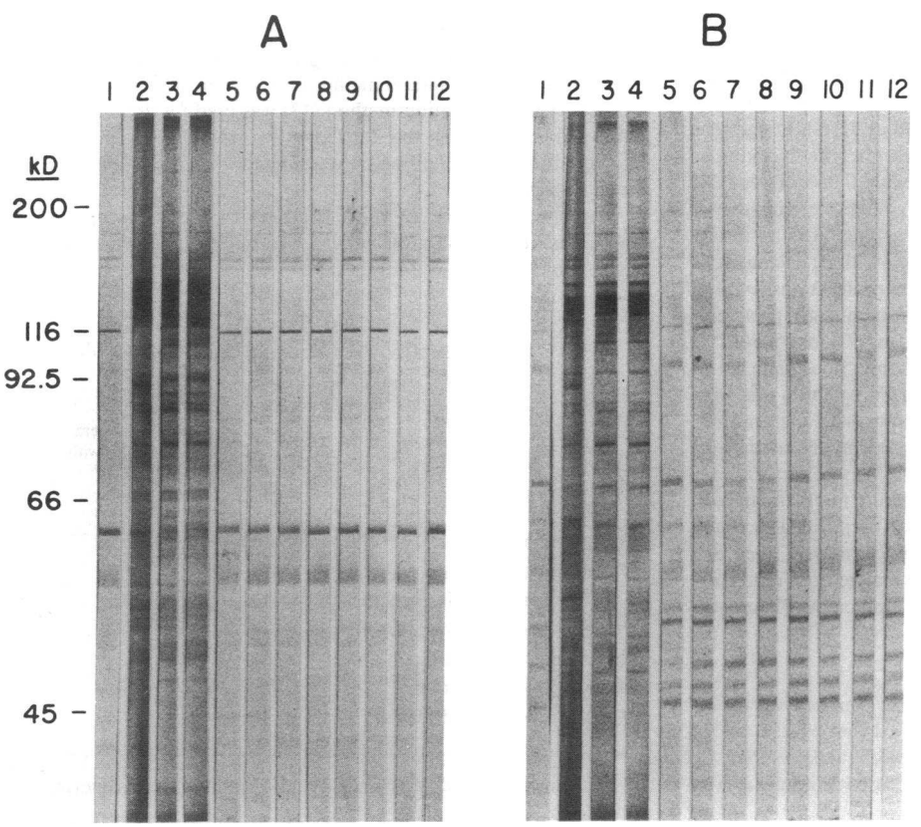

Figure 2. Demonstration of antigenic specificity of recombinant *O. volvulus* fusion proteins by immunoblot. Bacterial cell lysates from cells infected with OC 3.6 (*A*) and OC 9.3 (*B*) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose paper and developed with different human serum pools: lane *1*, nonendemic U. S. controls; lane *2*, *O. volvulus*-children (Nigeria); lane *3*, *O. volvulus*-adult (Nigeria); lane *4*, *O. volvulus*-adult (Guatemala); lane *5*, *B. malayi;* lane *6*, *W. bancrofti;* lane *7*, *A. lumbricoides;* lane *8*, *S. mansoni;* lane *9*, *M. ozzardi;* lane *10*, *L. loa;* lane *11*, *A. duodenale;* lane *12*, *D. medinensis.*

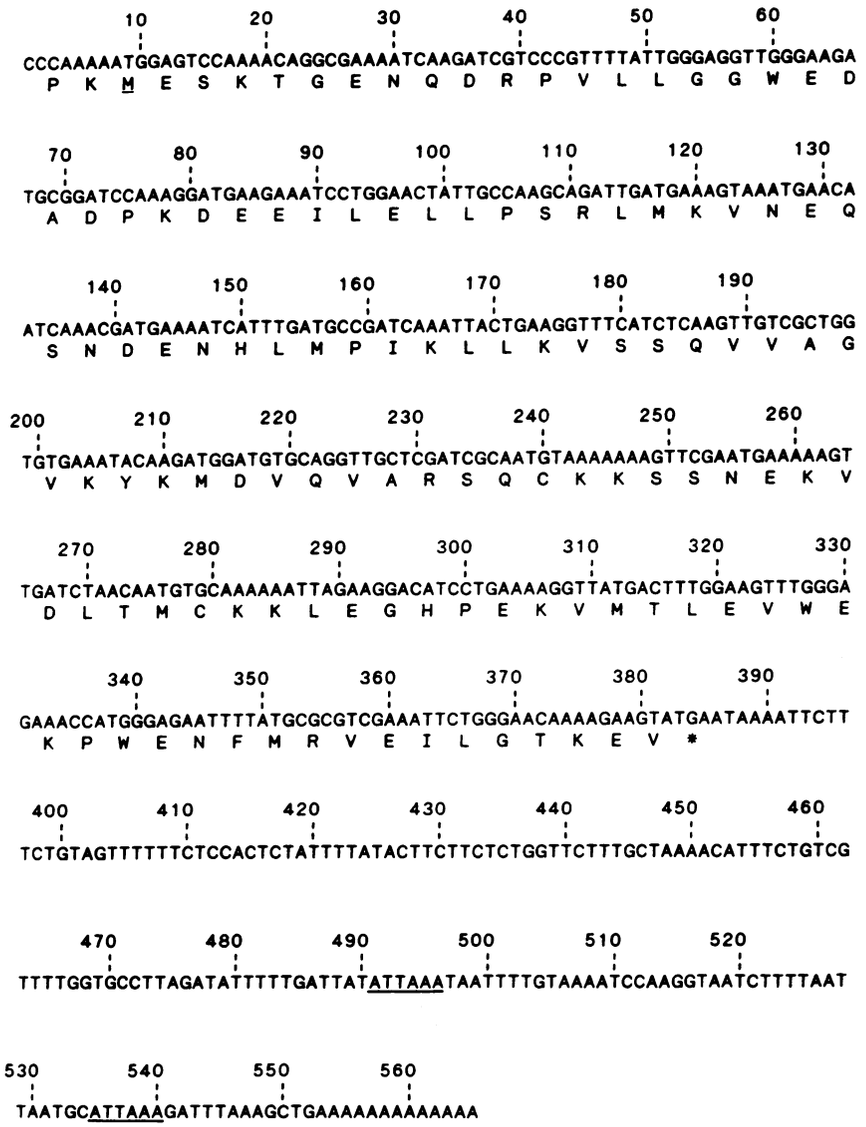

Figure 3. The sequence of the cDNA insert of clone OC 9.3 is shown (5′ to 3′) with the predicted amino acid sequence encoded by its open reading frame. The coding region begins with a methionine (M) codon 7 bp from the 5′ end and ends at base 382 with a stop codon (*). The consensus polyadenylation signals are underlined.

O. volvulus antigens may reflect exposure to parasite antigens without establishment of chronic infection (abortive infections). None of the nonendemic control sera tested contained detectable amounts of antibody to the protein products of OC 3.6 or OC 9.3.

Molecular characterization of clones OC 3.6 and OC 9.3. Western blot analysis showed that OC 3.6 and OC 9.3 produced fusion proteins with apparent molecular masses of 152

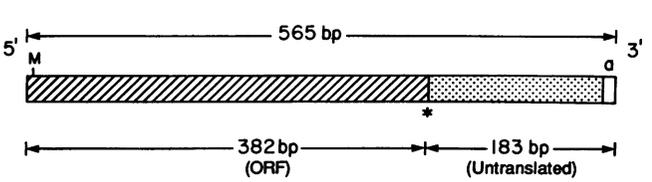

Figure 4. Schematic diagram of the OC 9.3 sequence. The cDNA insert has an open reading frame (*ORF*) of 382 bp and 183 bp of untranslated DNA at its 3′ end with a 12 bp poly-A tail (*a*).

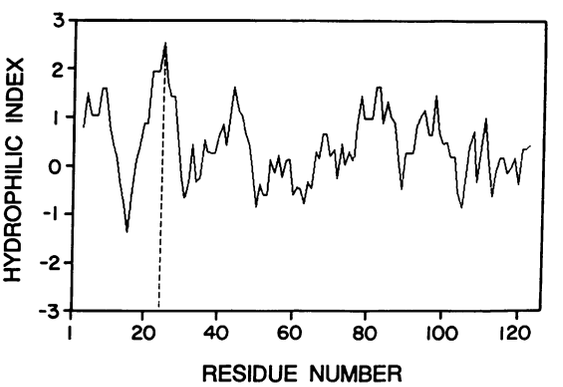

Figure 5. Hydropathy plot of the protein encoded by OC 9.3. Hydropathy analysis was performed by the method of Hopp and Woods. Hydropathy values were averaged for a window of six amino acid residues. Positive numbers indicate hydrophilicity. The point of highest hydrophilicity (between residues 22 and 27) is marked with a vertical dotted line.

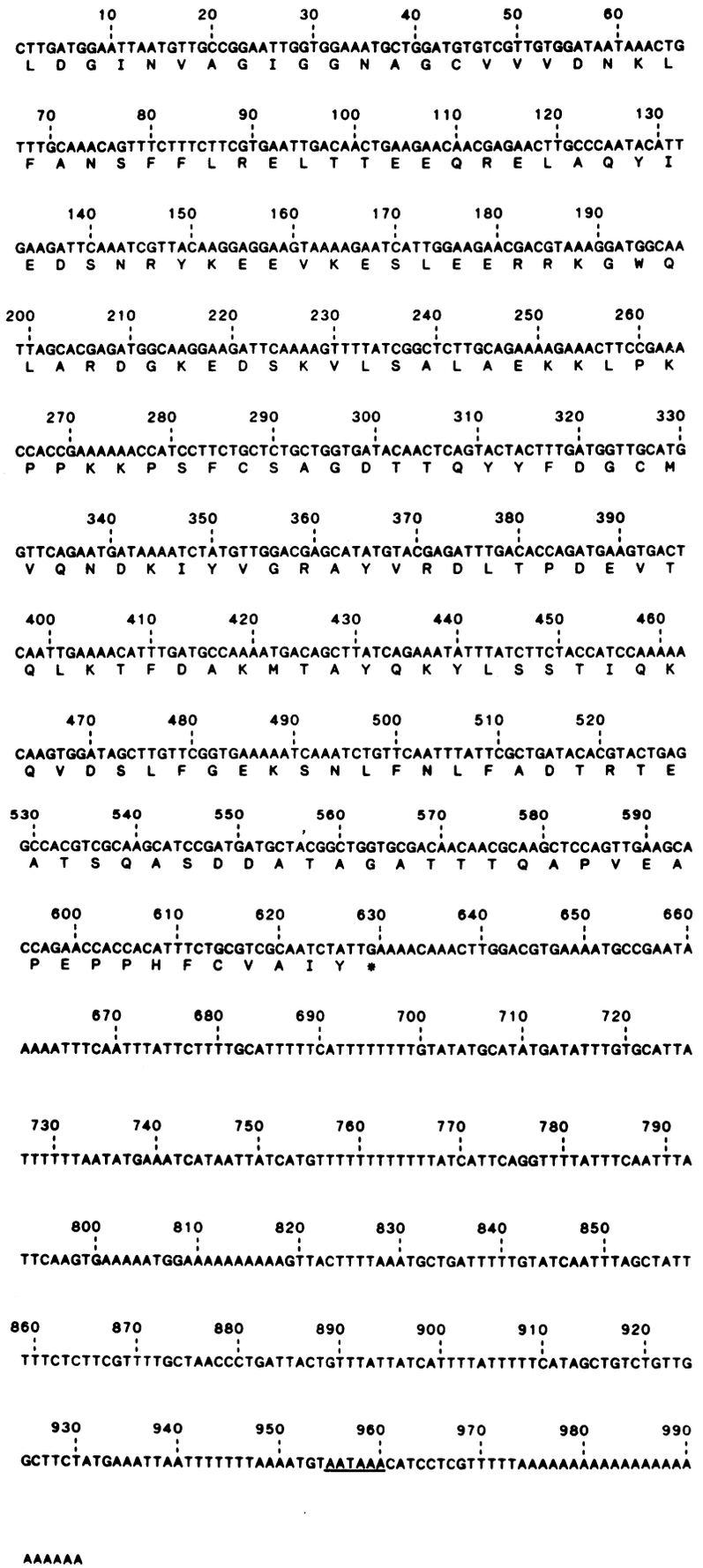

Figure 6. Sequence of the cDNA insert of clone OC 3.6. The complete nucleotide sequence of the insert is shown (5′ to 3′) along with the predicted amino acid sequence encoded by its long open reading frame. The indicated reading frame is in phase with the β-galactosidase gene of λ gt11. The stop codon is marked with an asterisk (*) and the polyadenylation signal is underlined.

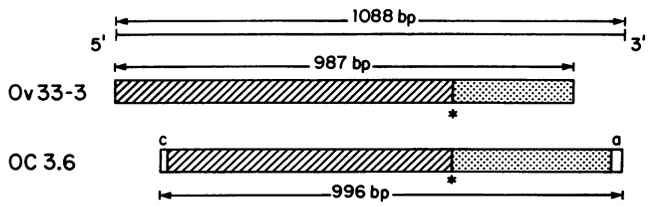

Figure 7. Relationship between clone OC 3.6 and a previously described clone, OV 33-3 (discussed in detail in Results). OC 3.6 contains 10 bp at the 5′ end of its open reading frame (*c*) and 100 bp in its 3′ untranslated region including a 23 bp poly-A tail (*a*) that are not present in OV 33-3.

and 138 kD as shown in Fig. 1. Species specificity of these recombinant antigens was demonstrated by Western blot analysis of serum pools from patients infected with a variety of other parasites (Fig. 2).

The DNA sequence of OC 9.3 is shown in Fig. 3.[2] A schematic diagram of the sequence is shown in Fig. 4. The insert contains 565 bp with an open reading frame (ORF) of 382 bp, and it appears to include the entire coding region for the protein. Although there is no untranslated DNA at the 5′ end of the sequence, we believe that the coding region begins with a methionine codon seven bp from the 5′ end with a purine in the −3 position (16). This sequence codes for a protein of 125 amino acids with a predicted molecular mass of 14.4 kD and a calculated pI of 5.09. The 3′ untranslated region of the insert contains 183 bp and ends with a 13-bp poly-A tail which begins 12 bp downstream from the sequence ATTAAA, a previously described variant of the eukaryotic consensus polyadenylation signal (17). Hydropathy analysis by the method of Hopp and Woods (18) predicts the protein to be predominantly hydrophilic (Fig. 5). None of the hydrophobic stretches is long enough to comprise a membrane-spanning α helix, and the sequence does not contain *N*-glycosylation sites. These features suggest a cytoplasmic location for the protein. Computer searches failed to reveal significant homology between the DNA or predicted amino acid sequence of OC 9.3 with previously reported sequences in the Genbank and NBRF data bases.

The 996-bp DNA sequence of OC 3.6 is shown in Fig. 6.[2] The sequence contains one 627-bp ORF, which begins at the 5′ end in phase with the reading frame of the β-galactosidase-gene of λ gt11, and 369 bp of untranslated DNA at the 3′ end. The protein encoded by the ORF contains 209 aminoacids and has a predicted molecular mass of 23.1 kD. OC 3.6 is closely related to an *O. volvulus* clone, OV 33-3, previously described by Lucius et al. (19). The sequence of OC 3.6 between bases 11 and 896 is identical to sequence in OV 33-3 (Fig. 7). OC 3.6 lacks 102 bp of sequence that are present at the 5′ end of the ORF of OV 33-3. However, OC 3.6 contains 10 bp at the 5′ end of its ORF and 100 bp in its 3′ untranslated region that are not present in OV 33-3. The latter segment contains a polyadenylation signal and a 23 bp poly-A tail that are not present in the published sequence of OV 33-3. A hydropathy plot of the composite sequence of OV 33-3 and OC 3.6 is shown in Fig. 8. The protein is predominantly hydrophilic. The most hydrophilic regions of the protein are in the shared portions of the sequences.

Discussion

Most attempts to develop antibody diagnostic tests for onchocerciasis have utilized native antigen which is in short supply and highly crossreactive with antigens from other nematode species (20). Several groups have reported that the specificity of such tests can be improved by using low molecular weight antigen fractions (21, 22) or by detecting antibodies of isotypes IgG_4 or IgE (23, 24). Despite these refinements, none of the assays based on native antigens has satisfactory sensitivity and specificity. Therefore, the goal of the present study was to clone and characterize *O. volvulus* cDNA clones that express recombinant antigens with immunodiagnostic potential. By careful use of well-defined serum pools from onchocerciasis patients and from patients infected with various other nematodes, we obtained seven *O. volvulus*-specific clones. Clones OC 9.3 and OC 3.6 were selected for additional studies because of their apparent diagnostic sensitivity and specificity in plaque-dot immunoblots with a large and varied panel of human sera. OC 9.3 codes for a low molecular weight *Onchocerca*-specific antigen with a predicted molecular mass of 14.4 kD. The larger apparent size of the fusion protein on SDS-PAGE may be due to the secondary structure of the protein. Indeed a hydropathy plot of the predicted amino acid sequence of this protein shows that it is quite hydrophilic, and a Chou-Fasman secondary structure analysis on the proposed sequence predicted extensive β turns (data not shown). No significant sequence homology was found between OC 9.3 and previously reported DNA or protein sequences. The 138-kD fusion protein produced by OC 9.3 was recognized by 83% of the onchocerciasis sera tested.

OC 3.6 is very closely related to OV 33-3, a previously described *O. volvulus* clone that was selected from the same cDNA library with a monospecific antibody raised against a 33-kD *O. volvulus* adult antigen (19). Although OC 3.6 lacks

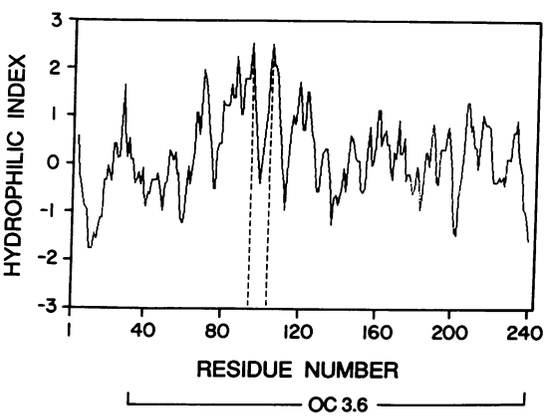

Figure 8. Hydropathy plot of the protein encoded by the composite DNA sequence of OV 33-3 and OC 3.6. Hydropathy analysis was performed by the method of Hopp and Woods. Hydropathy values were averaged for a window of six amino acid residues. Positive numbers indicate hydrophilicity. The two points of highest hydrophilicity (residues 58–63 and 68–73) are marked with vertical dotted lines.

2. Sequence data have been submitted to GenBank and assigned accession numbers M60280 (OC 9.3) and M60279 (OC 3.6).

102 bp at the 5′ end of the OV 33-3 sequence, it contains 10 bp at the 5′ end of its open reading frame and 100 bp in its 3′ untranslated region that are not in OV 33-3. The composite sequence contains 1,088 bp which is still not quite in agreement with the 1.2-kb transcript identified for this protein by Northern blot analysis (19). OC 3.6 produced a stable fusion protein that was recognized by antibodies in 54 of 55 sera from onchocerciasis patients tested. We have also found that this protein was recognized by sera from chimpanzees with prepatent and patent *O. volvulus* infections (authors' unpublished observations). This sensitivity was achieved in spite of the fact that the OC 3.6 fusion protein lacks the terminal 34 amino acids of the protein encoded by OV 33-3. This finding and the results of the hydropathy analysis suggest that the amino terminal end of OV 33-3 is not important in determining its antigenic reactivity. However, additional studies are needed to determine the immunodominant domains of this protein.

In addition to the clones described in this paper, a number of other recombinant *O. volvulus* antigens have been produced in other laboratories and proposed as immunodiagnostic antigens (19, 25, 26). Cooperative studies sponsored by the Filariasis Steering Committee of the TDR Program of the World Health Organization are in progress to determine the relative diagnostic potential of these candidate antigens (C. P. Ramachandran, personal communication). This cooperative approach should accelerate development of a sensitive, specific, and practical antibody diagnostic test that will be useful for diagnosis of onchocerciasis in endemic countries.

Acknowledgments

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