Echinococcus multilocularis: molecular and immunochemical characterization of diagnostic antigen II/3-10

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SUMMARY

A recombinant *Echinococcus multilocularis* antigen (II/3-10), which had previously been shown to exhibit immunodiagnostic characteristics highly specific for human alveolar echinococcosis, and the corresponding native parasite antigen, were further characterized with immunochemical and molecular biological methods. Immunoblot analysis using a polyclonal antiserum raised in rabbits against the recombinant protein, and subsequent Northern hybridization analysis, revealed that the native antigen was expressed by *E. multilocularis* at the adult as well as at the metacestode stage. In metacestodes, the antigen was shown by using indirect immunofluorescence and the same antiserum to be localized within the germinal layer and membrane structures of developing protoscolices. Electrophoretic analyses revealed remarkable differences in the apparent molecular weight of the antigen under reducing and non-reducing conditions. In further immunoblot analyses, anti-II/3-10 antibodies identified the corresponding epitopes on bands with identical M_r in all *E. multilocularis* isolates investigated (European, Asian and North American). By Southern hybridization analyses of the respective gene, phylogenetically related sequences were shown to be present in other helminth species such as *E. granulosus* and several *Taenia* spp. In the same respect, immunoblotting revealed that anti-II/3-10 antibodies reacted with antigens of different M_r from various *E. granulosus* isolates and some other cestode species, indicating the presence of shared and thus potentially cross-reacting epitopes. The relevance of these findings for the immunodiagnostic performance of the recombinant antigen is discussed.

Key words: *Echinococcus multilocularis*, Taeniid cestodes, immunodiagnosis, recombinant antigen.

INTRODUCTION

Alveolar echinococcosis is a zoonotic disease of man caused by infection with the larval stage (metacestode) of *Echinococcus multilocularis*. The parasite is found in its natural life-cycle only on the Northern hemisphere, where endemic areas include mainly central Europe, Canada and North America, China, Japan and others. Disease is attributed to the tumour-like invasive growth of the parasite located primarily in the liver of the patients. By the time the disease becomes clinically manifest, the lesions caused by the metacestodes have often reached an extension too large for complete surgical resection. Therefore, early serological detection of alveolar echinococcosis already in the stage of small hepatic lesions and subsequent treatment of patients are important means to reduce mortality.

A reliable immunodiagnosis of alveolar echinococcosis depends upon the availability of sufficient amounts of well-defined species-specific and diagnostically sensitive parasite antigens. In two former publications we summarized the identification and cloning of the gene fragment coding for the immunoreactive recombinant *E. multilocularis* antigen II/3 (Vogel *et al.* 1988) and the expression in bacteria of its subfragment II/3-10 carrying the relevant epitopes (Müller *et al.* 1989). Immunological evaluation of the recombinant antigen II/3-10 revealed a high diagnostic sensitivity and specificity (Gottstein *et al.* 1993) indicating a high potential value of this antigen for immunodiagnosis.

Besides the well-established diagnostic parameters of antigen II/3-10 only little was known about its biological nature. Therefore the present paper describes the characterization of the recombinant antigens II/3 and II/3-10 and the corresponding native parasite antigen with molecular biological and immunochemical methods.

MATERIALS AND METHODS

Parasites

Echinococcus multilocularis metacestode tissues were obtained from experimentally infected gerbils (*Meriones unguiculatus*), or C57BL/6J mice (parasite isolates corresponded in the main to those described previously by Gottstein & Mowatt (1991)) or from

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Table 1. Documentation on the parasite isolates used for immunoblotting, Southern and Northern hybridization analyses, respectively

Isolate code	Parasite species	Parasite stage	Geographical origin	Reference (remarks)	Host origin
CH6	*Echinococcus*	Metacestode	Switzerland	Gottstein & Mowatt (1991)	Gerbil
CH22	*multilocularis*	Metacestode	Switzerland	Gottstein & Mowatt (1991)	Gerbil
KF5 (clone)		Metacestode	France	Gottstein *et al.* (1992)	C57BL/6J
F4		Metacestode	France	Gottstein & Mowatt (1991)	Gerbil
A1		Metacestode	Austria	—	C57BL/6J
CDN1		Metacestode	Canada	Gottstein & Mowatt (1991)	Gerbil
SLI144		Metacestode	Alaska	Gottstein & Mowatt (1991)	*Microtus*
F4		Adult	France	—	Dog
Eg-h	*Echinococcus*	Metacestode	Switzerland	Horse liver cyst	Horse
Eg-c	*granulosus*	Metacestode	Switzerland	Cattle lung cyst	Cattle
Eg-s		Metacestode	Switzerland	Sheep lung cyst	Sheep
Eg-p		Metacestode	Switzerland	Pig liver cyst	Pig
Eg-d		Metacestode	Switzerland	Donkey liver cyst	Donkey
Tc	*Taenia crassiceps*	Larval	Egypt/Sudan		BALB/c
Th	*T. hydatigena*	Adult	Switzerland		Dog
Tsa	*T. saginata*	Adult	Switzerland		Human
Tso	*T. solium*	Larval	South Africa		Human
Tt	*T. taeniaeformis*	Adult	Switzerland		Cat
Me	*Moniezia expansa*	Adult	Switzerland		Cattle
Mc	*Mesocestoides corti*	Larval	Switzerland		BALB/c

naturally infected rodents caught on St Lawrence Island/Alaska (for details see Table 1). Adult stages of *E. multilocularis* were obtained from experimentally infected dogs after necropsy of the animals 24 days post-infection (p.i.) (dogs were perorally infected each with 12·5 g fertile metacestode tissue of the *E. multilocularis* isolate F4). Worms were recovered by incubating the resected small intestine in phosphate-buffered saline (PBS) at 37 °C for 1 h under slight agitation. Detached worms were aspirated from the sediment and washed 4 times in Hank's balanced salt solution (HBSS) at 37 °C prior to freezing at −80 °C. *E. granulosus* cyst walls and protoscolices were isolated from naturally infected horses, cows, pigs or sheep (all Swiss isolates). Other adult or metacestode-stage cestodes and other helminths were obtained as described previously (Gottstein & Mowatt, 1991). An overview list of all parasite materials used in this paper is provided in Table 1.

Reagents used for recombinant DNA techniques

Restriction endonucleases and *E. coli* DNA–Polymerase I (Kornberg polymerase and Klenow fragment) were obtained from Boehringer, Mannheim, FRG. T4 DNA ligase was purchased from New England Biolabs, Beverly, MA, USA. Sequenase 2·0-DNA sequencing kit and gene-clean DNA purification kit were supplied by United States Biochemical Corporation, Cleveland, OH, USA. Ultrapure reagents for RNA-Isolation were provided by Gibco BRL, Basel, CH.

Radioactive materials

Radioactive nucleotides [α^{32}P]dCTP and [α^{32}P]dATP for labelling of nucleic acids and [α^{35}S]dATP for sequence analyses were obtained from Dupon NEN, Regensdorf, CH.

Nucleic acids

Ultrapure deoxynucleotides and pd$(N)_6$ random primers for labelling of DNA probes were purchased from Pharmacia, Dübendorf, CH. DNA molecular weight standard 1 kbp ladder and RNA 0·24–9·5 kb ladder markers were obtained from New England Biolabs, Beverly, MA, USA, and from Gibco BRL, Basel, CH, respectively.

Recombinant DNA methods

All recombinant DNA methods, unless otherwise stated, were performed as described by Sambrook, Fritsch & Maniatis (1989).

Purification of nucleic acids

Genomic DNA from different parasite species was purified using the 'DNA extraction kit' obtained from Stratagene, Heidelberg, FRG, according to the manufacturer's protocols.

For the purification of total RNA a modification of the method described by Glišin, Crkvenjakov & Byus (1974) and Ullrich *et al.* (1977) was used.

Either 0·7 g of packed adult parasites or 3 g of metacestode tissue, respectively, were homogenized in 5 volumes of 4 M guanidinium-thiocyanate containing 100 mM Tris–HCl (pH 7·5) and 1% 2-mercaptoethanol. After homogenization, sodium lauryl sarcosinate was added to a final concentration of 0·5%. The samples were layered onto a cushion of 5·7 M CsCl/10 mM EDTA, pH 7·5 (ethylene-dinitrilo-tetraacetic acid) and ultracentrifuged for 24 h in a Beckman SW41 swinging bucket rotor at 32000 rpm. The pelleted total RNA was redissolved in diethyl pyrocarbonate (DEPC)-treated water.

Subcloning and sequencing

For determination of the nucleotide sequence of the cDNA inserts of clone II/3 and II/3-10, these were subcloned into the unique *Eco*RI site of plasmid vector pBluescript KS$^+$. Sequencing was performed with Sequenase 2·0 (United States Biochemical Corporation, Cleveland, OH, USA) and [^{35}S]dATP following the manufacturer's protocol. Sequencing primers KS and SK located adjacent to the polylinker region were used. DNA sequences were processed and derived amino acid sequences were compared to the SWISS-PROT sequence database (release 21) using the HUSAR computer program set (Heidelberg Unix Sequence Analysis Resources) of the German Cancer Research Center (DKFZ, Heidelberg, FRG).

Southern and Northern hybridizations

For Southern analysis, 20 μg aliquots of genomic DNA of different parasite species and isolates were digested with the appropriate restriction enzyme and electrophoresed through 0·8% agarose gels. RNA analysis was performed by separating 8 μg of total RNA on a 1·2% formaldehyde agarose gel (Lehrach *et al.* 1977). The samples were transferred to Nytran Nylon membranes (Schleicher & Schüll, Dassel, FRG) following the manufacturer's protocols. For preparation of a specific probe, the cDNA insert coding for II/3 was isolated and radiolabelled to high specific activity by random hexamer priming/extension reaction in the presence of [α^{32}P]dCTP or [α^{32}P]dATP (Feinberg & Vogelstein, 1983, 1984). Hybridization was performed in 50% formamide/6 × SSPE/0·5% SDS (sodium dodecyl sulphate) and 50% formamide/2·5 × Denhardt's reagent/5 × SSPE/0·1% SDS for Southern and Northern analysis, respectively. Following incubation for 12 h at 42 °C the filters were washed for 30 min with 6 × SSPE/0·1% SDS at room temperature, for 60 min at 37 °C with 1 × SSPE/0·1% SDS, and for 60 min at 65 °C with 0·1 × SSPE/1% SDS, if not otherwise stated. (1 × SSPE: 0·18 M NaCl/10 mM $NaPO_4$, pH 7·7/1 mM EDTA.)

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Parasite extracts were prepared from the respective parasite tissues as described previously (Gottstein, 1991). Aliquots of the protein extracts were mixed with an equal volume of 2 × sample buffer (100 mM Tris–HCl, pH 6·8/4% SDS/10% glycerol/0·1% bromophenol blue) containing 100 mM dithiothreitol (DTT) or not depending on the experiment. Samples were boiled for 5 min and separated by SDS–PAGE according to Laemmli (1970) using 4–20% pre-cast gradient gels (Bio-Rad, Glattbrugg, CH). Transfer to nitrocellulose was performed by the Western-blot technique (Towbin, Staehelin & Gordon, 1979). To accomplish the immunoblotting procedure, the filters were saturated with 3% bovine serum albumin (BSA) in PBS/0·1% Tween. Antisera were incubated with the filters at 200-fold dilution in PBS/0·1% Tween overnight at 4 °C. Bound antibodies were detected by anti-species antibodies conjugated to horseradish peroxidase (see below). The peroxidase-dependent colour reaction was carried out by incubating filters in 100 ml of staining solution (1 × PBS/1·5 mM 3,3′-diaminobenzidine tetrahydrochloride/0·003% H_2O_2).

Reagents used for immunological studies

Goat anti-rabbit IgG peroxidase-conjugate was purchased from Sigma, Buchs, CH (Cat. No. A 0545). The corresponding fluorescein-isothiocyanate (FITC)-conjugate was from Southern Biotechnology, Birmingham, AL, USA (Cat. 4010-02). Polyclonal hyperimmune serum directed against II/3-10 was obtained by immunization of rabbits with 100 μg II/3-10 antigen (purified according to the method of Müller *et al.* 1989) per injection, using RIBI® adjuvant and the immunization protocol provided by the manufacturer (Ribi Immunochem. Res., Hamilton, MO, USA).

Immunofluorescence microscopy

The direct immunofluorescence staining of the native II/3-10 antigen was performed on cryosections of hepatic liver lesions (8 μm thickness) from a rodent (*Microtus oeconomus*) naturally infected with *E. multilocularis* originating from St Lawrence Island, Alaska (this isolate was subsequently maintained in gerbils and designated as isolate SLI 144). Sections on glass slides were fixed in acetone, air dried and washed 3 times with PBS. One hundred μl of an incubation solution (Gottstein & Nash, 1991) containing 5 μl of hyperimmune rabbit serum or 5 μl of pre-immune rabbit negative control serum was dropped on the section and incubated for 30 min at 37 °C in a humid chamber. Slides were viewed in an Olympus BH-2 microscope and photographed on Kodak Ektachrome 64T film.

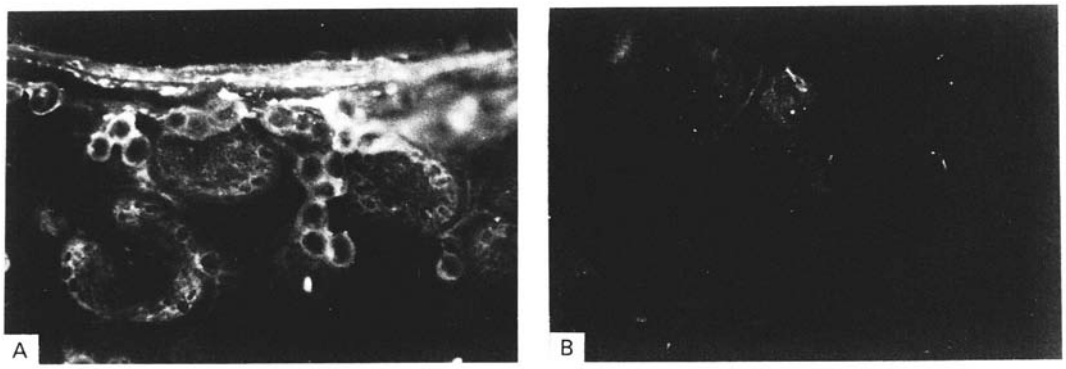

Fig. 1. Immunofluorescence analyses of cryosections from *Echinococcus multilocularis* metacestode tissue (liver lesion from a naturally infected rodent, see Materials and Methods section) using (A) rabbit hyperimmune serum against recombinant II/3-10 antigen. (B) Pre-immune negative control serum tested on a section of the same morphological area as in (A).

RESULTS

Immunofluorescence studies

In order to identify the localization of the II/3 antigen *in situ*, immunofluorescence studies using a polyclonal antiserum raised in rabbits against the affinity-purified recombinant II/3-10 antigen were performed. While no reaction above background was found with the pre-immune control serum (Fig. 1B), an intensive fluorescence was observed with the undifferentiated germinal layer and in the periphery of the individual cell conglomerates inside the protoscolices (Fig. 1A). Consequently the II/3 antigen seemed to be associated with membrane structures. Remarkably, no fluorescence was observed on the syncytial layer from the tegument of the developing protoscolices.

Stage specificity of the II/3 antigen

For the identification of the native II/3 antigen in parasite extracts, immunoblot analyses using the same rabbit hyperimmune serum were performed. Under reducing SDS–PAGE running conditions, anti-II/3-10 antibodies identified 2 molecules in metacestode extracts with apparent molecular weights of 65 kDa and 52 kDa (Fig. 2).

Additionally, we were able to detect bands with identical molecular weights in extracts of adult parasites suggesting expression of the II/3 antigen in both developmental stages. The protein of 52 kDa potentially could be interpreted as a processing or degradation product of the 65 kDa protein.

In contrast, marked differences in the apparent molecular weight of the II/3 antigen were observed under non-reducing SDS–PAGE running conditions (Fig. 2). A band of about 200 kDa was detectable in metacestodes as well as in the adult parasites. Speculatively, the reason for these differences could be explained by oligomerization of the II/3 antigen under native conditions or by a close association with other proteins. But this point deserves clarification by further investigations.

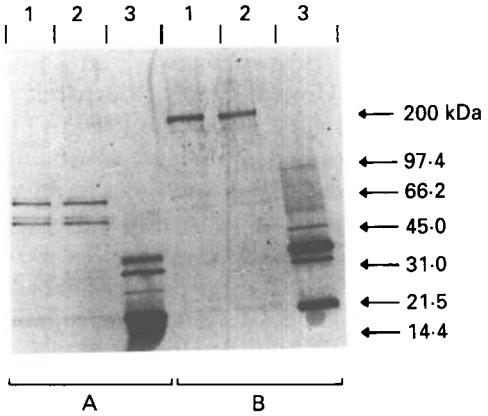

Fig. 2. Detection of *Echinococcus multilocularis* antigens with anti-II/3-10-specific hyperimmune serum raised in rabbits. Reducing SDS–PAGE running conditions (A) and non-reducing SDS–PAGE running conditions (B) were performed. Extracts of *E. multilocularis* metacestodes (Lanes 1), adults (Lanes 2), and extracts of *Escherichia coli* expressing recombinant II/3-10 antigen (Lanes 3) were analysed.

Species specificity of the II/3 antigen

Investigation of potential variability of the II/3 antigen within *E. multilocularis* isolates originating from geographically different areas was done by immunoblotting. No intra-species variation could be observed with respect to the II/3 antigen; all isolates studied showed the same banding pattern (data not shown).

In the enzyme-linked immunosorbent assay (ELISA) with the affinity-purified recombinant II/3-10 antigen, a weak immunological cross-reaction was observed with a few sera from patients with hydatidosis and cysticercosis (Gottstein *et al.* 1993). Therefore, extracts of *E. granulosus* metacestodes isolated from different intermediate

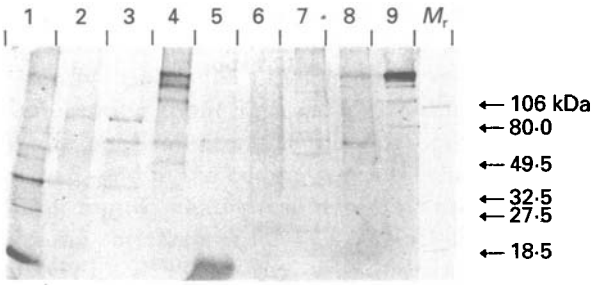

Fig. 3. Immunoblot with extracts of taeniid cestodes with II/3-10-specific hyperimmune serum. Eg-s (Lane 1), Eg-c (Lane 2), Eg-h (Lane 3), Eg-p (Lane 4), Th (Lane 5), Tso (Lane 6), Tsa (Lane 7), Mc (Lane 8), and CH6 (Lane 9). Molecular weight markers (M_r) are indicated. (For explanation of abbreviations refer to Table 1.)

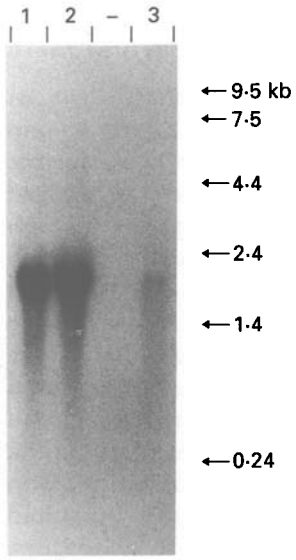

Fig. 4. Northern hybridization with radioactively labelled insert of clone II/3 as a probe. Total RNA isolated from *Echinococcus multilocularis* adults (EmAd, Lane 1) and metacestodes (KF5, Lane 2) and from *E. granulosus* metacestodes (Eg-c, Lane 3) was analysed. (For explanation of abbreviations refer to Table 1).

hosts and of different cestode species were analysed by immunoblotting with the anti-II/3-10 specific hyperimmune serum (Fig. 3). Bands of different molecular weights could be detected with the *E. granulosus* isolates and other cestode species reflecting the presence of cross-reacting epitopes.

Northern and Southern hybridizations

Further investigations with regard to these findings were performed at the level of nucleic acid. In Northern hybridizations using the radioactively labelled insert of clone II/3 as a probe, a single band of about 2·1 kb was found in total RNA of *E. multilocularis* metacestodes and adult parasites (Fig. 4). It is interesting to note that a transcript of the same size was observed in total RNA isolated from *E. granulosus* metacestodes but with a lower intensity of the signal. In immunoprecipitation following *in vitro* translation of total RNA in a cell-free rabbit reticulocyte extract, a single protein of about 65 kDa was precipitated from the translation mixture by the anti-II/3-10 antiserum both in metacestodes and adult parasites; a protein of 52 kDa was not observed (data not shown).

A uniform banding pattern could be observed in Southern hybridization with genomic DNA digested with *Eco*RI of several *E. multilocularis* isolates originating from geographically different areas. A band of about 5·5 kbp could be detected with the II/3 specific probe in all isolates studied (Fig. 5).

Consistent with the results of Northern analysis, cross-hybridizing bands were found with *E. granulosus* genomic DNA. All *E. granulosus* isolates studied, originating from different intermediate hosts, showed an identical banding pattern, differing from *E. multilocularis* with respect to a double band of about 2·5 and 2·8 kbp, respectively (Fig. 5).

With DNA of several other taeniid cestodes, no bands hybridizing with the II/3 specific probe were detectable under stringent washing conditions (final wash 65 °C, 0·1 × SSPE/1 % SDS), presumably due to decreased sequence homology to the *E. multilocularis* gene fragment (data not shown). In contrast, using conditions of low stringency (final wash room temperature, 1 × SSPE, 0·1 % SDS), cross-hybridization was clearly detectable with genomic DNA of *Taenia crassiceps*, *T. taeniaeformis*, and *T. saginata* (Fig. 6; to obtain a clearcut banding pattern, the enzyme *Bam*HI was chosen for digestion of the genomic DNAs). Furthermore, weak signals were observed with *T. hydatigena* and *T. solium*, indicating the widespread distribution of the II/3 gene or related sequences in the family *Taeniidae*. No signal was detectable with *Moniezia expansa* and *Mesocestoides corti*.

Sequences of clones II/3 and II/3-10

The clone II/3 and its derivative II/3-10 carried cDNA inserts of 1 kbp and 0·6 kbp, respectively (Müller *et al.* 1989). Each insert was isolated, subcloned into pBluescript KS$^+$, and its respective nucleotide sequence determined. Both strands were completely sequenced. The primary DNA sequences and the derived amino acid sequences of the two clones in question are shown in Fig. 6.

An open reading frame extends over most of the nucleotide sequence of clone II/3. An amber stopcodon (TAG) at position 886 is followed by a 3′ non-coding region of 190 bp and a poly-A tail of 28 A-residues. The underlined ATTAAA sequence element found 21 bp upstream of the poly-A tail presumably serves as a polyadenylation signal.

The sequence of the cDNA of truncated clone II/3-10, which was generated by sonication of the

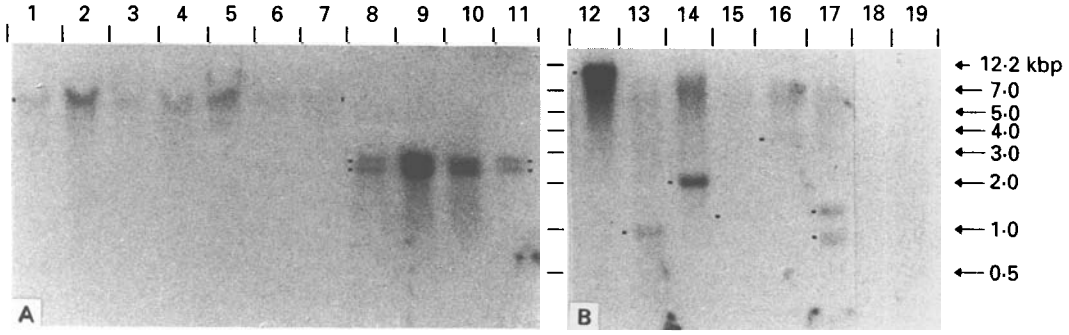

Fig. 5. Southern analyses of genomic DNA with radioactively labelled insert of clone II/3 as a probe. (A) Digestion with *Eco*RI and conditions of high stringency (Lanes 1–11); (B) restriction with *Bam*HI and low stringency (Lanes 12–19). CH6 (Lane 1), CH22 (Lane 2), A1 (Lane 3), F4 (Lane 4), KF5 (Lane 5), CND1 (Lane 6) and SLI144 (Lane 7); isolates Eg-c (Lane 8), Eg-h (Lane 9), Eg-p (Lane 10) and Eg-d (Lane 11); CH6 (Lane 12), Tc (Lane 13), Tt (Lane 14), Th (Lane 15), Tso (Lane 16), Tsa (Lane 17), Mc (Lane 18) and Me (Lane 19). (For explanation of abbreviations refer to Table 1.)

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GAATTCCGGTTTTTTTTTTTCTTGGTGGAAAAATCCAAGATTAACAAGCGCATTTTGGCATTGTGTACTGGCAAC    75
          F  F  F  F  L  V  E  K  S  K  I  N  K  R  I  L  A  L  C  T  G  N

CATGAGCTCTACATGCGTAGAAGAAAGTCAGACTCTATTGAGGTGCAACAGATGAAGATTCAGGCCAAGGAGGAA   150
 H  E  L  Y  M  R  R  R  K  S  D  S  I  E  V  Q  Q  M  K  I  Q  A  K  E  E

CGTGAATTGAAGGAGGCTGAGAGACAACGCCTGAAGGAGGAACGATTGCAACGTATGGAAAATGAACAGAAACTG   225
 R  E  L  K  E  A  E  R  Q  R  L  K  E  E  R  L  Q  R  M  E  N  E  Q  K  L

CGGGAGCTTCGTGCTCAAATGGTCGAAAAGGAGTCTGACTTAGCGGATATGAAGAATAAGGCATCTGCCTATGAG   300
 R  E  L  R  A  Q  M  V  E  K  E  S  D  L  A  D  M  K  N  K  A  S  A  Y  E

AGTAAGATTGCGGAGCTGGAGATGCTGCTACAGCAGGAGCGACATGCGCGTGAGAGTCTTCAGAAGAGCCAAGAC   375
 S  K  I  A  E  L  E  M  L  L  Q  Q  E  R  H  A  R  E  S  L  Q  K  S  Q  D

AAACTGGCGGAGATGAACAGAAAGCTGAAGGAGGAGACTGCGGCATCAGCCGAAGAGCGCGACCGTCTGATGGCC   450
 K  L  A  E  M  N  R  K  L  K  E  E  T  A  A  S  A  E  E  R  D  R  L  M  A

CAGCGTGACGAAGTGCAACGCGAAGTTGAGGCTCAGAAGGTCGCCATGGCCAAGAAGGAAGCTGAAAAGGCTCAG   525
 Q  R  D  E  V  Q  R  E  V  E  A  Q  K  V  A  M  A  K  K  E  A  E  K  A  Q
                                                ▼
GCTGAAGCTGAGCTTCGCAGAATGCGTGAGAAACACGATGCAAAGCACAAGTCCCAGGTCAATGGCAGTGGTGAC   600
 A  E  A  E  L  R  R  M  R  E  K  H  D  A  K  H  K  S  Q  V  N  G  S  G  D

GCTGCTTCGCAGGATGATGAAAGTGAAGCCAAGGAACTTGAGGTGATACCAAATGTGAGGCGGACGGAGGAATCG   675
 A  A  S  Q  D  D  E  S  E  A  K  E  L  E  V  I  P  N  V  R  R  T  E  E  S

AGGGTGACGGCCGTCTCTAAGAATGAGACGCTCCAGACGAAGCTGGCCAACCTCAAAATGGAGTTGAGCTCGACA   750
 R  V  T  A  V  S  K  N  E  T  L  Q  T  K  L  A  N  L  K  M  E  L  S  S  T

CGCGATCAGTCGAAAATGCGCGACATTGATCGTCGTCATGAGTACAATGTGCGGGAGGGTAATGACAAGTACAAG   825
 R  D  Q  S  K  M  R  D  I  D  R  R  H  E  Y  N  V  R  E  G  N  D  K  Y  K

ACACTGCGCAACATTCGCAAGGGCAACACCATGTGTCGTGTTGAACAGTTTGAGTCGATGTAGAAATGTTACAGT   900
 T  L  R  N  I  R  K  G  N  T  M  C  R  V  E  Q  F  E  S  M  *

TGTCTTCATTCCCCTCATCTTTCTGCAATTTTGGACCCTCTATCACTACGGTTACTTCCTCAATCATGCTGCTAC   975

AGTGCTATCCAACATTCCATTTTTATTCTCGCGATGCACACCTGTCTTTTCATTCTGCCCCTTCTTATCTAACGT  1050

CCACTTTATTAAACTGCTATCGTACGCTAAAAAAAAAAAAAAAAAAAAAAAAAAAACCGGAATTC            1115
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Fig. 6. Nucleotide and deduced aminoacid sequences of *Echinococcus multilocularis* cDNA-clone II/3. Adaptor sequences containing *Eco*RI sites used for cloning are boxed. Presumable polyadenylation signal ATTAAA is underlined. Sequence of truncated clone II/3-10 is shaded in grey. Last nucleotide (G at position 574) of II/3-10 indicated by an arrow head is followed by adaptor sequences (not shown).

cDNA-insert encoding antigen II/3 and subsequent cloning of the shortened fragment, is located in the 5′-terminal region of clone II/3 ending at position 575. The coding capacities of the two cDNAs are 255 and 188 aminoacids, potentially yielding proteins of 29·5 kDa and 21·5 kDa, respectively. This is consistent with the size observed for the corresponding recombinant antigens (Vogel *et al.* 1988; Müller *et al.* 1989).

In order to establish a possible function for the antigen, the deduced amino acid sequence of clone II/3 was compared to the sequences of the SWISS-

PROT data base. It proved to be identical to the sequence of the *E. multilocularis* antigen Em10 recently published by Frosch *et al.* (1991), representing about 52% of its C-terminal sequence. While only minor nucleotide changes at the ultimate 5′-end in the coding region of the II/3-cDNA were found, that presumably could be due to cloning artefacts (TTT.TTT.TTT instead of GAG.TTT.TCT; Fig. 6), extensive differences concerning the 3′-non-coding region of clone II/3 compared to the sequence published by Frosch *et al.* (1991) were observed. The II/3 clone ends prior to the last 27 nucleotides of the non-coding region showing no homology to the corresponding sequence published for Em10. This raises the possibility of cDNA clones resulting from transcripts of different copies of the respective gene.

DISCUSSION

In two former studies the identification of an immunodiagnostic recombinant *E. multilocularis* antigen II/3 (Vogel *et al.* 1988) and the expression in bacteria of its subfragment II/3-10 carrying the relevant epitopes (Müller *et al.* 1989) have been described. The recombinant antigen allowed detection of anti-*E. multilocularis* antibodies in human patients with high diagnostic sensitivity and specificity (Gottstein *et al.* 1993). Although the immunodiagnostic performance of the antigen had been studied already in detail and its suitability for immunodiagnosis of alveolar echinococcosis is well established, almost nothing was known about its biological nature. But, generally, characterization of antigens used for immunological diagnosis exceeding diagnostic evaluation is desirable. The purpose of the present study therefore was the analysis of the recombinant antigen and the corresponding native parasite antigen with immunological and molecular-biological methods.

Nucleotide sequence analyses of the cDNAs coding for II/3 and II/3-10 revealed the identity of their sequences with a section of the sequence published for antigen Em10, an *E. multilocularis*-antigen which was described by Frosch *et al.* in 1991. The clone II/3 represented about 52% of its C-terminal end, clone II/3-10 was located at the 5′ end of clone II/3.

In immunoblot anti-II/3-10 antibodies identified two molecules in metacestode extracts with apparent molecular weights of 65 kDa and 52 kDa. Two bands with about the same molecular weights have been observed in metacestode extracts using an antiserum specific for antigen Em10 (65 and 55 kDa, respectively; Frosch *et al.* 1991).

Based on the nucleotide sequence analysis and the fact that anti-II/3-10 (as shown in our study) and Em10-specific antiserum (Frosch *et al.* 1991) detected molecules in the same molecular weight range, it seems reasonable to assume that both antigens are identical although the complete sequence of the gene represented by clone II/3 is still required for confirmation.

Immunoblot analysis using a II/3-10-specific antiserum raised in rabbits against the affinity-purified recombinant antigen and Northern hybridization analysis with a specific probe revealed expression of the antigen in metacestodes as well as in adult parasites. The level of expression seems to be similar in both developmental stages. This novel finding could reflect an important function of the antigen, independent of the developmental stage.

All isolates of *E. multilocularis* originating from geographically different areas showed the same banding pattern in immunoblot with the specific hyperimmune serum. Clone II/3-10 was originally isolated from a cDNA library constructed from metacestode tissue of a Swiss isolate (CH-14; Vogel *et al.* 1988). Cross-reaction of anti-II/3-10 antibodies with antigens from parasite material of different geographical origin was consistent with the reactivity of patient sera from the corresponding regions with the recombinant II/3-10 antigen (Müller *et al.* 1989).

Immunoblot analyses with the anti-II/3-10 antiserum clearly demonstrated the presence of cross-reacting epitopes in *E. granulosus* and several *Taenia* species. These findings contributed to the explanation for the weak immunological cross-reaction observed with a few sera from patients with hydatidosis and cysticercosis in the ELISA with recombinant II/3-10 antigen (Gottstein *et al.* 1993). Nevertheless, immunological reactivity of sera from patients with respective diseases seemed to be directed mainly against epitopes differing from those recognized by hyperimmune antibodies, thus finally resulting in an appropriate specificity of the recombinant II/3-10 antigen for the immunodiagnosis of alveolar echinococcosis.

The results concerning the immunological characterization of the II/3-antigen could be confirmed by experiments at the nucleic acid level. Southern hybridization showed that DNA sequences hybridizing with a II/3-specific probe were present in *E. granulosus* and several *Taenia* species. While the II/3 related sequences in *E. granulosus* seemed to be relatively conserved compared to the II/3-gene of *E. multilocularis*, homology of the corresponding sequences in the different *Taenia* species was less prominent.

Frosch *et al.* (1991) demonstrated the Em10 antigen by immunoblot analysis in *E. multilocularis* but not in *E. granulosus*. Therefore they suggested a possible role for this antigen in infiltrative tumour-like growth of *E. multilocularis* metacestodes. In addition to *E. multilocularis* we were able to detect the II/3 antigen in *E. granulosus* and some *Taenia* species which do not demonstrate chronic invasive

growth characteristics such as observed in alveolar echinococcosis. Provided that both antigens are identical, the novel findings presented in this publication are in contradiction. Therefore experiments are now underway to isolate and analyse the complete II/3-gene from *E. multilocularis* and from *E. granulosus* for comparative characterization.

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