Comparative analysis of full-length antigen II/3 from *Echinococcus multilocularis* and *E. granulosus*

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(*Received 8 November 1993; revised 10 February 1994; accepted 10 February 1994*)

SUMMARY

The recombinant *Echinococcus multilocularis* antigen II/3-10 is one of the most promising tools for immunodiagnosis of alveolar echinococcosis in human patients. Its nucleic acid sequence represents a part of the *E. multilocularis* gene encoding the metacestode antigen II/3, the former being basically present and expressed in both *E. multilocularis* and *E. granulosus*. Most (94%) patients with alveolar echinococcosis respond to infection with a marked anti-II/3-10 IgG synthesis; in contrast, most of the cystic echinococcosis patients do not, for some reason, recognize the recombinant antigen. We tackled this problem by generating cDNA derived from both *E. granulosus* and *E. multilocularis* full length II/3 genes, performed by reverse transcription and PCR amplification. Sequence analysis revealed a very high degree of conservation of the primary sequence of the antigen II/3 in both *Echinococcus* species. cDNA fragments were subcloned and expressed in *E. coli* as fusion proteins with *Schistosoma japonicum* glutathione S-transferase. Recombinant proteins were affinity purified and comparatively assessed by ELISA with respect to antibody-binding characteristics. Sera from patients suffering from cystic echinococcosis showed no significant differences in reactivity with the antigens derived from either *E. multilocularis* or *E. granulosus*. Therefore, parameters other than some minor differences in the primary sequence seem to be responsible for the lack of antigen II/3 recognition in cystic echinococcosis.

Note: Nucleotide sequence data reported in this paper have been submitted to the GenBank® data base with the accession numbers U05573 and U05574.

Key words: *Echinococcus multilocularis*, *Echinococcus granulosus*, taeniid cestodes, immunodiagnosis, PCR, recombinant antigen II/3, GST-fusion protein, ELISA.

INTRODUCTION

Echinococcosis in humans is caused by a proliferating metacestode following infection with different species of the genus *Echinococcus*. Alveolar echinococcosis (*E. multilocularis*) and cystic echinococcosis (*E. granulosus*) are both of medical and public health importance. While *E. granulosus* has a cosmopolitan distribution, *E. multilocularis* is found in its natural life-cycle only in the Northern hemisphere.

Cystic echinococcosis is characterized by well-delimited cysts formed most frequently in the liver, but also in the lungs and other organs; such hydatid cysts can be removed completely by surgery in most cases. In contrast, alveolar echinococcosis is caused by the tumour-like invasive growth of the parasite, affecting primarily the liver and then other organs by infiltration or metastasis. By the time the disease becomes clinically manifest, the lesions caused by the *E. multilocularis* metacestode are often too large for complete surgical resection. Therefore, early serological detection of alveolar echinococcosis at the stage of small hepatic infiltrates followed by appropriate surgical treatment are important means to reduce mortality (Ammann *et al.* 1990; Gottstein, 1992).

Immunological evaluation of the recombinant *E. multilocularis* antigen II/3-10 (Vogel *et al.* 1988; Müller *et al.* 1989) revealed a high diagnostic sensitivity and specificity (Gottstein *et al.* 1993), thus indicating a high potential value of this antigen for immunodiagnosis.

Recently we characterized this recombinant antigen II/3-10 and the corresponding native metacestode antigen using molecular biological and immunochemical methods (Felleisen & Gottstein, 1993). These analyses revealed the presumed identity of antigen II/3-10 with an antigen Em10 recently described by Frosch *et al.* (1991), which seemed to be related to the antigen EM4 described by Hemmings & McManus (1991). Southern and Northern hybridization analyses and immunoblotting using a specific anti-II/3-10 hyperimmune serum raised in rabbits demonstrated that sequences encoding antigen II/3-10 seemed to be present not only in *E. multilocularis*, but also in *E. granulosus*, and that related antigens were expressed in both species, respectively (Felleisen & Gottstein, 1993).

Although also synthesized by *E. granulosus*, very few patients (6%) with cystic echinococcosis had serum antibodies against the recombinant II/3-10 antigen (Gottstein *et al.* 1993). Possibly, the antigens from both *Echinococcus* species might differ in their

primary sequence, thus serum antibodies from patients with the two diseases might be directed against epitopes differing from those recognized by hyperimmune antibodies. Alternatively, the different reactivities could be caused by other parameters such as differences in post-translational modifications or accessibility or presentation of the antigen to the immune system during the course of infection.

In this paper we describe the PCR directed cloning and comparative sequence analysis of cDNA fragments encoding the full-length antigens from both *Echinococcus* species, their expression in bacteria as recombinant antigens, and the comparative immunological evaluation of these antigens using the sera of alveolar and cystic echinococcosis patients.

MATERIALS AND METHODS

Bacterial strains and parasites

Bacterial strain DH5α was used for propagation of plasmid vector pBluescript KS$^+$, subcloning in pGEX B, and expression of GST-fusion proteins. *Echinococcus multilocularis* metacestode tissue (clone KF5; Gottstein *et al.* (1992)) was obtained from experimentally infected C57BL/6J mice. *E. granulosus* brood capsules were isolated from a fertile lung cyst from a naturally infected cow (Swiss isolate) provided by Dr E. Lanz from the municipal abattoir of Berne.

Reagents used for recombinant DNA techniques

Restriction endonucleases and *E. coli* DNA-polymerase I (Klenow fragment) were obtained from Boehringer, Mannheim, Germany. T4 DNA ligase and MuLV reverse transcriptase were 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, Switzerland. Recombinant RNasin® ribonuclease inhibitor was obtained from Promega, Zürich, Switzerland and native Taq DNA polymerase from Perkin Elmer Cetus, Basel, Switzerland.

Radioactive material

Radioactive nucleotides α^{35}S-dATP for sequence analyses were supplied by Dupon NEN, Regensdorf, Switzerland.

Nucleic acids

Ultrapure desoxynucleotides and p(dT)$_{15}$ primer for cDNA synthesis were purchased from Pharmacia, Dübendorf, Switzerland, and Boehringer, Mannheim, Germany, respectively. Primer GST1 (5′-GCATGGCCTTTGCAGGG-3′) used for sequencing DNA-fragments cloned in pGEX B was obtained from Synthecell Corp. (Rockville, MD. USA).

Primers RB1 (5′-AAA.CAT.ATG.TTG.AAG.AGG-3′) and RB2 (5′-AGA.GGA.TCC.AAA.ATT.GC-3′) used for polymerase chain reaction (PCR) were synthesized through TIB MOLBIOL, Berlin, Germany. Plasmid pBluescript KS$^+$ and sequencing primers SK and KS were provided by Stratagene, Zürich, Switzerland. As DNA molecular weight standard, bacteriophage λ DNA was digested with restriction enzymes *Eco*R I and *Hin*d III.

Expression vector pGEX B, a derivative of pGEX-3X (Smith & Johnson, 1988) containing a multiple cloning site with additional recognition sequences for *Bgl* II and *Hin*d III (C. Paranhos, unpublished results), was kindly provided by C. Paranhos, bioMérieux, Marcy-l'Étoile, France.

Recombinant DNA methods

All recombinant DNA methods were carried out according to Sambrook, Fritsch & Maniatis (1989) unless otherwise stated.

Purification of total RNA

For the purification of total RNA a modification of the method described by Glisin, Crkvenjakov & Byus (1974) and Ullrich *et al.* (1977) was used. Three g of *E. multilocularis* metacestode tissue and 0·4 g of packed brood capsules from *E. granulosus*, respectively, were homogenized in 5 volumes of 4 M guanidinum-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, 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. The total amount of RNA as determined spectrophotometrically was about 750 μg for *E. multilocularis* and 80 μg for *E. granulosus*.

Reverse transcription

Total RNA was transcribed into single-stranded cDNA following the protocol of Frohman (1990). Four μg RNA were heated for 3 min at 65 °C, quenched on ice, and transcribed in a total volume of 20 μl of 1 × transcription buffer (50 mM Tris–HCl, pH 8·15, 6 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, each dNTP at 1·5 mM) containing 10 U RNasin® ribonuclease inhibitor, 0·5 μg p(dT)$_{15}$ primer, and 12·5 U MuLV reverse transcriptase. Following incubation for 1 h at 42 °C and 30 min at 52 °C, 80 μl

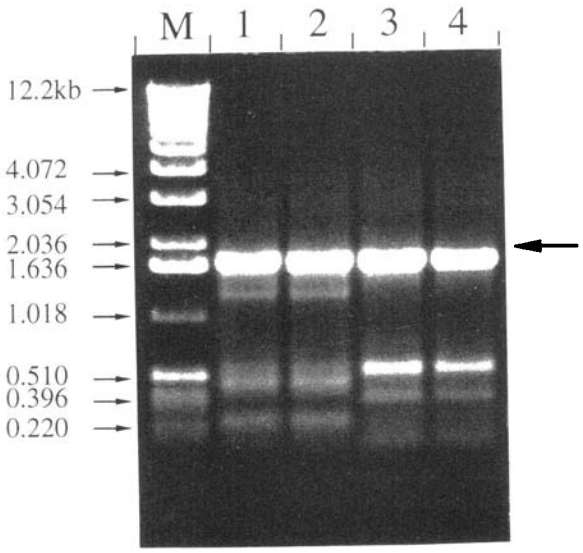

Fig. 1. PCR amplification of full-length antigen II/3 cDNA. Electrophoretic analysis of amplification products on 1 % agarose gel. Molecular weight standards $\lambda \times$ *Eco*R I/*Hin*d III (Lane M); amplification from *Echinococcus multilocularis* (Lanes 1 and 2) and from *E. granulosus* RNA (Lanes 3 and 4). The results of two independent experiments, respectively, are shown.

of DEPC-treated water were added. The resulting 'cDNA-pool' was used as starting material for PCR amplification.

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was carried out in a 50 μl reaction volume of 1 × PCR buffer (Perkin Elmer Cetus) using 10 μl of first strand cDNA-pool as template, 25 pmol of primers RB1 and RB2, each dNTP at 200 μM final concentration, and 2 U native Taq polymerase. Samples were overlaid with mineral oil and amplified in a Perkin Elmer Cetus thermal cycler using the following temperature profile. Denaturing: 94 °C, 45 s; annealing: 45 °C, 25 s; extension: 72 °C, 3 min. Following 30 cycles a final extension step was added for 15 min at 72 °C.

Subcloning and sequencing

PCR amplification products were isolated from 1 % agarose gels using a gene-clean DNA purification kit (USB). Isolated fragments were cloned into the unique *Eco*R V site of plasmid vector pBluescript KS$^+$. For sequencing, deletion mutants were generated by subcloning appropriate restriction fragments in pBluescript KS$^+$. Sequencing was performed with Sequenase 2·0 (USB) and α-^{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 using the GCG-computer program set for VAX/VMS computers (Devereux, Haeberli & Smithies, 1984).

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

Protein samples were mixed with an equal volume of 2 × sample buffer (100 mM Tris–HCl, pH 6·8/4 % SDS/ 10 % glycerol/ 10 % 2-mercaptoethanol, 0·05 % bromophenol blue), boiled for 5 min and separated by SDS–PAGE according to the method of Laemmli (1970) on 8 × 12 cm gels (Hoefer Scientific Instruments, Littau, Switzerland). Gels were either stained with Coomassie blue or used for immunoblotting. Transfer of proteins 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/ 0·3 % Tween. Antisera were incubated with the filters at varying dilutions in PBS/ 0·3 % Tween overnight at 4 °C. Bound antibodies were detected by recombinant protein G (Zymed) conjugated to horseradish peroxidase at 1000-fold dilution for 1 h and developed using the enhanced chemiluminescence system (ECL, Amersham, Zürich, Switzerland).

Reagents used for immunological studies

For detection of GST fusion proteins, polyclonal hyperimmune sera directed against antigen II/3-10 (Felleisen & Gottstein, 1993) and against *Fasciola hepatica* protease Fcp1 fused to GST (Heussler & Dobbelaere, 1994) were used. The antibodies were affinity purified against recombinant II/3-10 antigen and the GST carrier protein, respectively, using the method described by Olmsted (1981).

Horseradish peroxidase conjugated with recombinant protein G was purchased from Zymed, San Francisco, CA, USA (Cat. No. 10-1223). Alkaline phosphatase-labelled goat anti-human IgG for ELISA was obtained from Sigma, Buchs, Switzerland. Enhanced chemiluminescence detection kit was provided by Amersham, Zürich, Switzerland.

Human sera

For ELISA tests, sera of 28 European patients with clinically and histologically proven alveolar echinococcosis and 30 patients suffering from cystic echinococcosis, respectively, were taken from a collection of sera used in a previous study (Gottstein *et al.* 1993). Sera from 30 healthy individuals served as negative controls.

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) including setting of test parameters were performed basically as described previously (Gottstein *et al.*

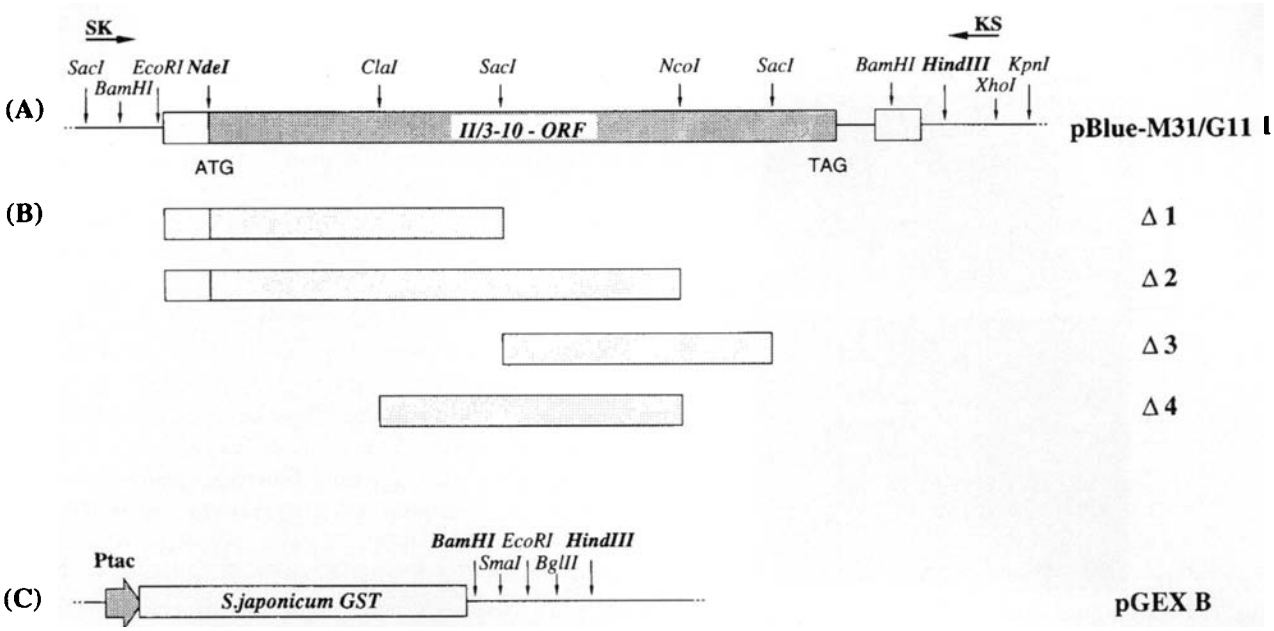

Fig. 2. Partial restriction map of cDNA clones pBlue M31 and pBlue G11 (A), schematic drawing of the subclones (Δ1–4) used for determination of the respective nucleotide sequences (B) and schematic illustration of the restriction sites contained in the multiple cloning site of pGEX B (C). Sequences originating from primers RB1 and RB2 used for PCR amplification are drawn as open boxes. Arrows labelled KS and SK represent sequencing primers located in the polylinker region of pBluescript KS⁺. Ptac = tac promoter.

1993). ELISA plates (Nunc maxisorb 96F, Nunc, Roskilde, Denmark) were sensitized with purified recombinant antigens at concentrations of 1 μg/ml.

Absorbance was measured at 405 nm using a Dynatech MR7000 reader coupled to a Macintosh Centris 600 computer with the Biocalc® software (Dynatech, Embrach, Switzerland).

Expression and affinity purification of GST-fusion proteins

Small-scale expression and screening of transformants of GST fusion proteins was done according to the method described by Smith & Johnson (1988). For large-scale production of recombinant antigens, overnight cultures of *E. coli* strain DH5α transformed with parental or recombinant pGEX B plasmids were diluted 1:10 in 500 ml of fresh medium and grown for 75 min at 37 °C under vigorous shaking. Then isopropyl-β-D-thiogalactopyranosid (IPTG) was added to a final concentration of 1 mM and bacteria were grown for an additional 2–6 h, depending on the antigen expressed. Cells were pelleted and resuspended in 25 ml of PBS/1 % Triton X–100. Bacteria were lysed by mild sonication and sedimented at 10000 ***g*** at 4 °C for 10 min. The pellet ('insoluble fraction') was discarded, the supernatant ('soluble fraction') was used for affinity purification of GST fusion proteins.

Appropriate amounts of the supernatant were mixed with glutathione Sepharose® 4B beads (Pharmacia LKB, Dübendorf, Switzerland). Following intensive washing, bound antigens were eluted using 5 mM reduced glutathione (Sigma) in 50 mM Tris–HCl, at a final pH of 7·5. For ELISA assays the antigens were dialysed overnight against several large volumes of coating buffer (50 mM sodium carbonate, pH 9·6) at 4 °C.

RESULTS

Cloning of cDNA fragments using polymerase chain reaction (PCR)

Reverse transcription carried out on RNA of *E. granulosus* and *E. multilocularis* followed by PCR using primers RB1 and RB2, derived from the 5′- and 3′-sequences of cDNAs encoding *E. multilocularis* antigens Em10 and II/3-10, resulted mainly in populations of cDNA fragments of approximately 1750 bp with both species (Fig. 1), corresponding to the size of the open reading frame published for *E. multilocularis* clone pEM10 by Frosch *et al.* (1991). Amplification products were isolated from preparative agarose gels and cloned into the unique *Eco*R V site of pBluescript KS⁺.

The 5′- and 3′-ends of the resulting clones were sequenced using primers SK and KS located in the polylinker region of pBluescript KS⁺. Following restriction analysis, different appropriate restriction enzyme fragments were subcloned into pBluescript KS⁺ and their respective nucleotide sequences determined (for a schematic overview see Fig. 2B). Various clones from different amplification reactions were analysed in parallel, to eliminate errors introduced by the Taq DNA polymerase itself.

The resulting combined DNA sequences and the derived amino acid sequences are shown in Fig. 3.

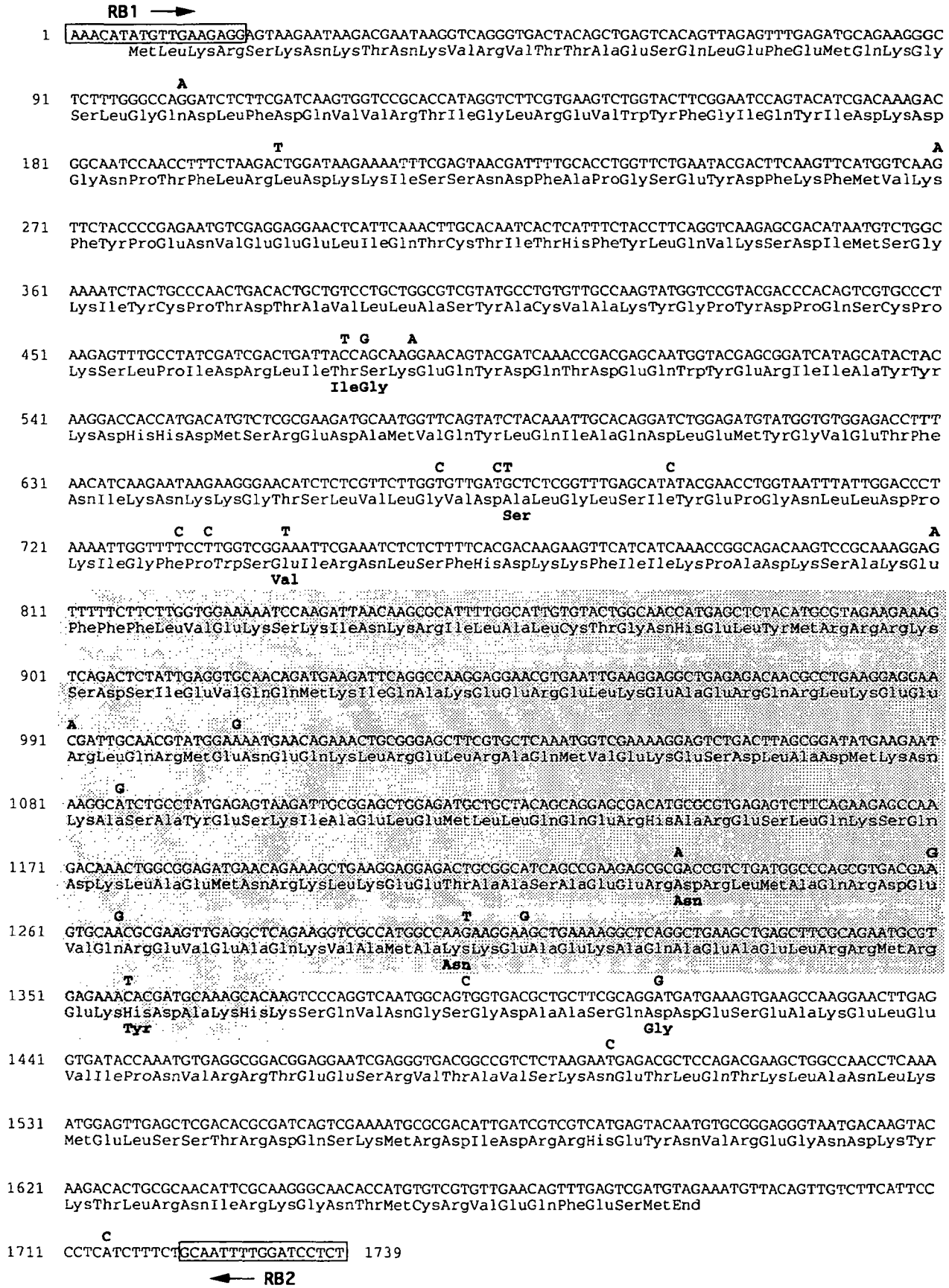

Fig. 3. Combined nucleotide and deduced amino acid sequences of different *Echinococcus multilocularis* cDNA-clones. Nucleotides different in the corresponding sequence of *E. granulosus* are depicted in bold type above the respective sequence of *E. multilocularis*, differences on the level of amino acids are depicted below. Sequences derived from the primers RB1 and RB2 used for PCR amplification are boxed. Area of the antigen fragment II/3-10 is shaded in grey.

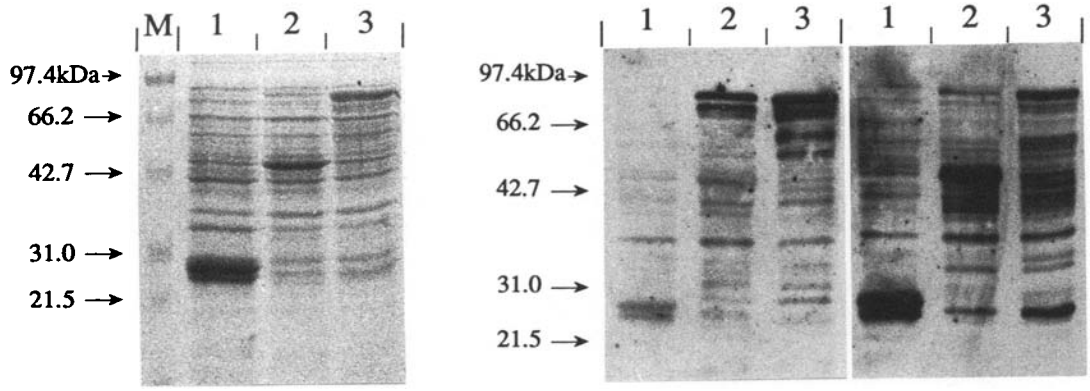

Fig. 4. Expression of *Echinococcus multilocularis* and *E. granulosus* antigens as fusion proteins with *Schistosoma japonicum* glutathione S-transferase (GST). Total bacterial extracts were boiled with sample buffer and subjected to SDS–PAGE on 15% gels. Representative clones expressing *E. multilocularis* (Lane 2) and *E. granulosus*-derived antigens (Lane 3) and GST carrier protein (Lane 1) are shown. Molecular weight standards (Lane M). Coomassie blue staining (A), and immunoblotting using affinity-purified α-II/3-10 (B) and α-GST specific rabbit hyperimmune sera (C) was performed.

Comparison of the nucleotide sequences derived from the two *Echinococcus* species revealed 1 nucleotide exchange in the 3′-non-coding region and a total of 25 differences with respect to the coding region leading to 98·45% homology at the nucleotide level. The nucleotide exchanges result in 8 differences in the amino acid sequences leading to 98·57% homology at the amino acid level.

Expression of GST-fusion proteins

E. multilocularis and *E. granulosus* full length cDNA sequences represented by two clones termed pBlue-M31 and pBlue-G11, respectively, were subcloned into the expression vector pGEX B using the *Hind* III recognition sequence of the polylinker of pBluescript KS$^+$ which was ligated into the respective site of pGEX B, and following treatment with *E. coli* DNA polymerase I (Klenow fragment) the *Nde* I restriction site included in the sequence of amplification primer RB1, which was ligated to the blunt ended *Bam*H I restriction site of pGEX B, respectively (Fig. 2A and C), resulting in an in-frame fusion to the *Schistosoma japonicum* glutathione S-transferase gene.

Several clones containing the desired sequences were identified, and their nucleotide sequence at the region of fusion to GST was determined for confirmation. Expression of GST-fusion proteins was monitored by SDS–PAGE and immunoblotting using an anti-II/3-10 specific rabbit hyperimmune serum (Felleisen & Gottstein, 1993). Bacteria transformed with the recombinant plasmids produced fusion proteins of approximately 89 kDa as calculated from the deduced amino acid sequences (Fig. 4). While in the case of the *E. granulosus*-derived clones the fusion protein of 89 kDa represented an abundant synthesis product, only minor amounts were seen in the *E. multilocularis*-derived clones. Here, the most abundant protein found had an apparent molecular weight of approximately 43 kDa, representing a product of degradation at the 3′-end of the fusion protein: while affinity-purified antibodies directed against the GST carrier protein recognized this protein strongly (Fig. 4C), almost no reaction was seen with affinity-purified antibodies directed against II/3-10 (Fig. 4B). Identical patterns were observed with different clones of identical genomic origin (data not shown). Two clones termed pGEX-M31 and pGEX-G11 were selected for further characterization.

Affinity purification of GST-fusion proteins

In order to produce the recombinant antigens in amounts and in purity sufficient for ELISA studies, large-scale cultures (500 ml) of bacteria transformed with the parental and the recombinant plasmids were set up. While large amounts were obtained from cultures expressing the GST carrier protein and the *E. granulosus*-derived fusion protein GST-II/3-G11, expression of the *E. multilocularis*-derived fusion protein GST-II/3-M31 was unsatisfactory (Fig. 5). Therefore, the conditions of induction had to be optimized for this particular antigen. Best results were obtained using short induction times (2 h) and a large number of small-scale cultures (4 ml) instead of one large-scale culture (data not shown).

Affinity purification of the antigens is shown in Fig. 5. Reasonable amounts of the GST-II/3-G11 fusion protein and the GST carrier protein were contained in the soluble fraction and could be purified easily by adsorption to glutathione Sepharose®-4B beads. On the other hand, most of the GST-II/3-M31 fusion protein was found in the

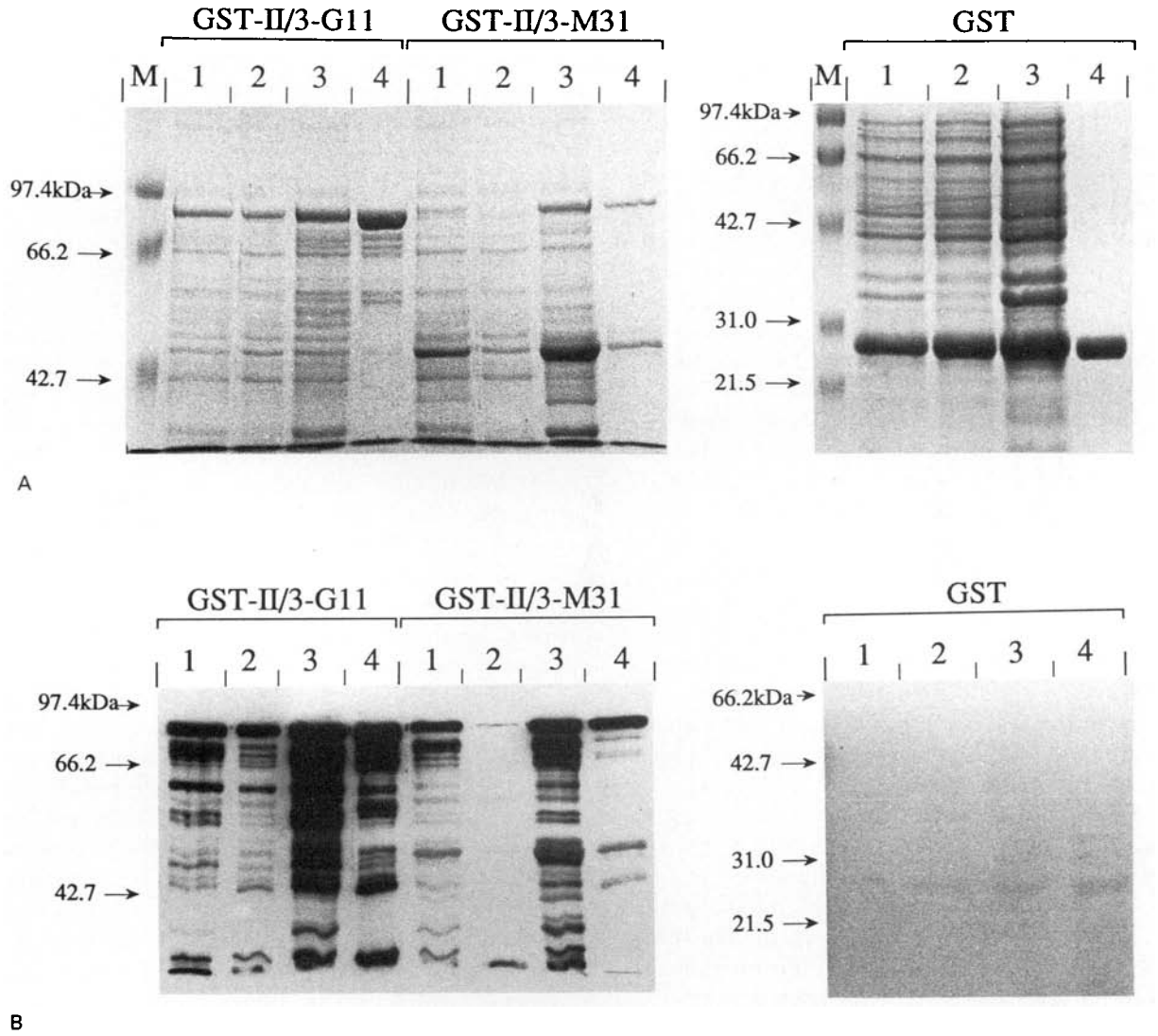

Fig. 5. Affinity purification of recombinant antigens. Total bacterial extracts (Lane 1), soluble fractions (Lane 2), insoluble fractions (Lane 3) and proteins bound to glutathione Sepharose beads (Lane 4) were analysed. Molecular weight standard (Lane M). Coomassie blue staining (A), and immunoblotting using affinity purified α-II/3-10 specific rabbit hyperimmune serum (B) was performed. Samples containing antigens GST-II/3-G11 and GST-II/3-M31 were separated on 8% SDS-PAGE gels, samples containing GST carrier protein on 12% gels.

insoluble fraction. Therefore, only limited amounts of this antigen could be purified. The antigens were eluted from the beads by competition with free reduced glutathione and, following dialysis, adjusted to a concentration of approximately 1 μg/ml. The 43 kDa degradation product of antigen GST-II/3-M31 proved to bind more tightly to the purification matrix compared to the full-length product resulting in an enrichment of the latter during elution (data not shown).

Enzyme-linked immunosorbent assay (ELISA)

Affinity-purified recombinant antigens GST-II/3-G11 and GST-II/3-M31 and the GST carrier (control) protein were used to sensitize ELISA plates. Antigens derived from two independent purification procedures were used in each case and were tested with sera from patients with proven alveolar and cystic echinococcosis, respectively, and with sera from healthy blood donors.

No significant differences between homologous antigens derived from two independent purification batches were observed (data not shown), therefore, the mean values of the two tests were used for further considerations. Absorbance values from minor cross-reactions with the purified GST carrier protein were corrected by subtraction from the value obtained with the recombinant full-length II/3-antigen.

The reactivities of the *E. multilocularis* and *E. granulosus* patient sera with the two different antigens in ELISA were compared by calculation of the

correlation coefficient for the respective absorbance values. A value of $r = 0{\cdot}982$ was obtained (Fig. 6).

DISCUSSION

Immunodiagnosis of alveolar echinococcosis represents one of the important tools for the detection of infection and disease. The immunodiagnostic *E. multilocularis* recombinant antigen II/3-10 allowed detection of anti-*E. multilocularis* antibodies in human patients with high diagnostic sensitivity and specificity (Gottstein *et al.* 1993). Although homologous antigens are also synthesized by *E. granulosus* (Felleisen & Gottstein, 1993), very few sera from patients (6%) with cystic echinococcosis had serum antibodies against the recombinant II/3-10 antigen (Gottstein *et al.* 1993).

Therefore, the purpose of the present study was to isolate and to analyse the complete antigen II/3-gene from *E. multilocularis* and from *E. granulosus*, and to express the respective sequences in *E. coli* as recombinant antigens for a comparative immunological characterization and a potential elucidation of the phenomenon of humoral responsiveness and non-responsiveness to the antigen of patients suffering from alveolar or cystic echinococcosis, respectively.

Isolation of full-length cDNA clones was accomplished by the polymerase chain reaction (PCR). Besides the major amplification products representing full-length fragments, minor smaller amplification products were observed. The latter were probably due to unspecific priming during amplification, because no hybridization was observed in Southern blot analyses using radiolabelled II/3-10 cDNA-fragment as a probe (data not shown), although the possibility cannot be ruled out that they are reflecting alternative splicing of the II/3-mRNA especially in *E. granulosus*.

Nucleotide sequence analyses of the cDNAs obtained from *E. multilocularis* and from *E. granulosus* revealed a considerable high degree of conservation between both species. Only 8 out of 559 amino acids were different, resulting in approximately 98·6% sequence identity. This conservation may suggest a relatively important functional role of the antigen in both *Echinococcus* species. No differences concerning the nucleotide sequence of the *E. multilocularis* cDNA clones compared to the published sequences were observed.

The sequence identity of full length antigen II/3 gene (98·6%) was in the same range as those published for antigens Em13/Eg13 (98·29%; Frosch *et al.* 1993) supporting the concept of a close genetic relationship between both *Echinococcus* species.

Despite the high degree of conservation in their primary sequence the recombinant antigens expressed as GST-fusion proteins in *E. coli* behaved rather differently. While the *E. granulosus*-derived protein GST-II/3-G11 was expressed in high amounts and mostly in an undegraded soluble form, the synthesis of the *E. multilocularis*-derived fusion protein GST-II/3-M31 was only very limited and preferentially contained in the insoluble fraction in a degraded form.

Although Smith & Johnson (1988) reported that a high percentage of GST-fusion constructs were at least partly soluble, insolubility of foreign proteins expressed in *E. coli* is very common (Marston, 1986). The factors influencing solubility and stability of GST-fusion proteins are not well characterized, but the charge and size of the respective protein seem to play an important role (Smith & Johnson, 1988). Calculation of the isoelectric point from the deduced amino acid sequences revealed values of 7·48 and 7·98 for the *E. multilocularis* and *E. granulosus* antigens, respectively. But whether this difference could account for the differences in behaviour of the two proteins was not clear. Nevertheless, both antigens were successfully affinity purified from bacterial extracts and used for comparative immunological characterization in ELISA.

Sequence analyses revealed that the differences in the primary sequence of antigens II/3 between both *Echinococcus* species were only very limited. But the regions of divergence could potentially represent the most important antigenic regions of the respective proteins. If this would be true, sera from patients with cystic echinococcosis showing no or only low reactivity with antigen II/3 from *E. multilocularis* should recognize the respective protein from *E. granulosus* considerably more strongly. But no significant differences in the reactivity of sera from patients either suffering from alveolar or from cystic echinococcosis were observed with the antigens derived from both *Echinococcus* species. A comparative investigation with the purified GST-fusion proteins in ELISA demonstrated statistically very good correlations ($r = 0{\cdot}982$). Differences in the mean A_{405} nm values of the sera with the two antigens ($0{\cdot}340 \pm 0{\cdot}294$ and $0{\cdot}209 \pm 0{\cdot}201$ for *E. granulosus* and *E. multilocularis*, antigens, respectively) were due to slight variations in technical parameters (e.g. antigen concentration) resulting in a slope of the regression line in Fig. 6 of 0·668.

Sera of healthy blood donors were used to calculate a threshold value for determining positive reactions for both antigens (mean + 2 S.D.). In this way, only one serum of each patient group was considered borderline negative with the heterologous antigen and positive with the respective homologous antigen (sera pointed by arrow heads in Fig. 6). These findings strongly suggested that the primary sequence of the antigens was not the crucial cause for the different reactivity of sera from patients with respective diseases.

Nevertheless, the possibility could not be ruled out that conformational epitopes contained in the

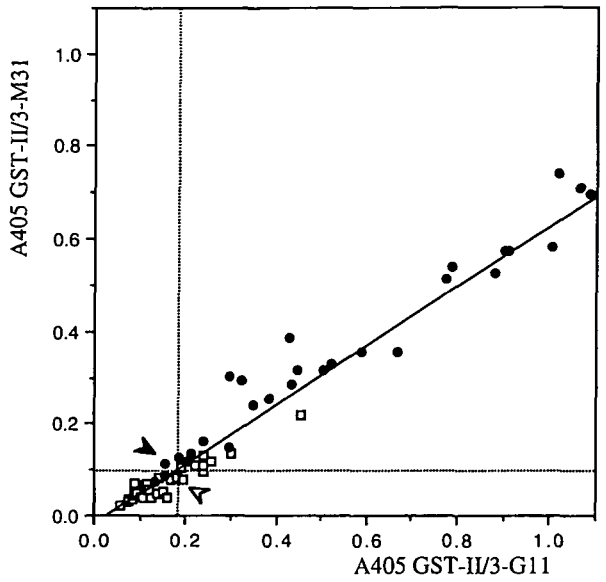

Fig. 6. Direct comparative analysis by ELISA of purified recombinant antigen GST-II/3-M31 of *Echinococcus multilocularis* versus antigen GST-II/3-G11 of *E. granulosus*. The investigation was performed using defined sera of *E. multilocularis* patients ($n = 28$) and of *E. granulosus* patients ($n = 30$). The reactivity of sera of healthy blood donors ($n = 30$) was used to determine threshold values for each antigen (tv = mean + 2 S.D.). ●, *E. multilocularis* patient sera; □, *E. granulosus* patient sera.

variable regions played an important role for the immunological reactivity of the antigens. In computer analyses of the two proteins, no significant differences with respect to hydrophilicity, flexibility and surface probability profiles were observed. Furthermore, the calculated secondary structure of both proteins proved to be very similar (data not shown). Therefore, it was reasonable to assume that the two recombinant fusion proteins had a very similar secondary structure and that as well linear and conformational epitopes were identical or at least very similar.

Antigen II/3 obviously is localized in the undifferentiated germinal layer of metacestode tissue (Felleisen & Gottstein, 1993). Alveolar echinococcosis is characterized by the infiltrative tumour-like growth of the parasite with parasite cell protrusions being probably in direct contact with the host tissue and therefore accessible for the host immune system (Mehlhorn, Eckert & Thompson, 1983). During cystic echinococcosis, in contrast, well-delimited cysts are formed which are encapsulated by host connective tissue surrounding a thick laminated layer, and thus seem to be somehow more inert to recognition by the host immune system and to subsequently induced effector mechanisms. Therefore, antigen II/3 could be less accessible in cystic echinococcosis, being hypothetically presented to the immune system only in the case of rupture or leakage of cysts. Ongoing studies are focusing on the potential of antigen II/3 to detect *E. granulosus* cyst damage by serological means.

Other reasons which may help to explain the divergent immunological phenomenon described above include post-translational modifications or processing of the antigen as well as species-specific immune regulatory or modulating mechanisms. These points as well as the actual function of antigen II/3 *in vivo* deserve further clarification.

We thank Ms V. Zimmermann for her expert technical assistance, Dr A. Busato for his support with respect to the statistical evaluation, Dr P. Jacquier for the supply of human sera and Dr E. Lanz for providing bovine *E. granulosus* hydatid cysts. This work was supported by a grant obtained from the Swiss National Science Foundation (No. 31-29651.90).

REFERENCES

AMMANN, R. W., HIRSBRUNNER, R., COTTING, J., STEIGER, U., JACQUIER, P. & ECKERT, J. (1990). Recurrence rate after discontinuation of long-term mebendazole therapy in alveolar echinococcosis (preliminary results). *American Journal of Tropical Medicine and Hygiene* **43**, 506–15.

DEVEREUX, J. R., HAEBERLI, P. & SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the Vax. *Nucleic Acids Research* **12**, 387–95.

FELLEISEN, R. & GOTTSTEIN, B. (1993). *Echinococcus multilocularis*: molecular and immunochemical characterization of diagnostic antigen II/3-10. *Parasitology* **107**, 335–42.

FROHMAN, M. A. (1990). Rapid amplification of cDNA ends (RACE): user friendly cDNA cloning. *Amplifications* **5**, 11–15.

FROSCH, P. M., FROSCH, M., PFISTER, T., SCHAAD, V. & BITTER-SUERMANN, D. (1991). Cloning and characterization of an immunodominant major surface antigen of *Echinococcus multilocularis*. *Molecular and Biochemical Parasitology* **48**, 121–30.

FROSCH, P. M., GEIER, C., KAUP, F.-J., MÜLLER, A. & FROSCH, M. (1993). Molecular cloning of an echinococcal microtrichal antigen immunoreactive in *Echinococcus multilocularis* disease. *Molecular and Biochemical Parasitology* **58**, 301–10.

GLISIN, V., CRKVENJAKOV, R. & BYUS, C. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* **13**, 2633.

GOTTSTEIN, B. (1992). Molecular and immunological diagnosis of echinococcosis. *Clinical Microbiological Reviews* **5**, 248–61.

GOTTSTEIN, B., DEPLAZES, P. & AUBERT, M. (1992). *Echinococcus multilocularis*: immunological study on the 'Em2-positive' laminated layer during *in vitro* and *in vivo* post-oncospheral and larval development. *Parasitology Research* **78**, 291–7.

GOTTSTEIN, B., JACQUIER, P., BRESSON-HADNI, S. & ECKERT, J. (1993). Improved primary immunodiagnosis of alveolar echinococcosis (*Echinococcus multilocularis* infection of humans) by Em2^{plus}-ELISA. *Journal of Clinical Microbiology* **31**, 373–6.

HEMMINGS, L. & MCMANUS, D. P. (1991). The diagnostic value and molecular characterisation of an *Echinococcus multilocularis* antigen gene clone. *Molecular and Biochemical Parasitology* **44**, 53–62.

HEUSSLER, V. T. & DOBBELAERE, D. A. E. (1994). Cloning of a protease gene family of *Fasciola hepatica* by polymerase chain reaction. *Molecular and Biochemical Parasitology* **64**, 11–23.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature, London* **227**, 680–5.

MARSTON, A. O. (1986). The purification of eukaryotic polypeptides synthesized in *Escherichica coli*. *The Biochemical Journal* **240**, 1–12.

MEHLHORN, H., ECKERT, J. & THOMPSON, R. C. A. (1983). Proliferation and metastases formation of larval *Echinococcus multilocularis*. II. Ultrastructural investigations. *Zeitschrift für Parasitenkunde* **69**, 749–63.

MÜLLER, N., GOTTSTEIN, B., VOGEL, M., FLURY, K. & SEEBECK, T. (1989). Application of a recombinant *Echinococcus multilocularis* antigen in an enzyme-linked immunosorbent assay for immunodiagnosis of human alveolar echinococcosis. *Molecular and Biochemical Parasitology* **36**, 151–60.

OLMSTED, J. B. (1981). Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *Journal of Biological Chemistry* **256**, 11955–7.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

SMITH, D. B. & JOHNSON, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene* **67**, 31–40.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, USA* **76**, 4350–4.

ULLRICH, A., SHINE, J., CHIRGWIN, J., PICTET, R., TISCHER, E., RUTTER, W. J. & GOODMAN, H. M. (1977). Rat insulin genes: construction of plasmids containing the coding sequences. *Science* **196**, 1313.

VOGEL, M., GOTTSTEIN, B., MÜLLER, N. & SEEBECK, T. (1988). Production of a recombinant antigen of *Echinococcus multilocularis* with high immunodiagnostic sensitivity and specificity. *Molecular and Biochemical Parasitology* **31**, 117–28.