Presence of polydnavirus transcripts in an egg–larval parasitoid and its lepidopterous host

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The parasitoid *Chelonus inanitus* (Braconidae, Hymenoptera) oviposits into eggs of *Spodoptera littoralis* (Noctuidae, Lepidoptera) and, along with the egg, also injects polydnaviruses and venom, which are prerequisites for successful parasitoid development. The parasitoid larva develops within the embryonic and larval stages of the host, which enters metamorphosis precociously and arrests development in the prepupal stage. Polydnaviruses are responsible for the developmental arrest and interfere with the host’s endocrine system in the last larval instar. Polydnaviruses have a segmented genome and are transmitted as a provirus integrated in the wasp’s genome. Virions are only formed in female wasps and no virus replication is seen in the parasitized host. Here it is shown that very small amounts of viral transcripts were found in parasitized eggs and early larval instars of *S. littoralis*. Later on, transcript quantities increased and were highest in the late last larval instar for two of the three viral segments tested and in the penultimate to early last larval instar for the third segment. These are the first data on the occurrence of viral transcripts in the host of an egg–larval parasitoid and they are different from data reported for hosts of larval parasitoids, where transcript levels are already high shortly after parasitization. The analysis of three open reading frames by RT–PCR revealed viral transcripts in parasitized *S. littoralis* and in female pupae of *C. inanitus*, indicating the absence of host specificity. For one open reading frame, transcripts were also seen in male pupae, suggesting transcription from integrated viral DNA.

Introduction

Polydnaviruses are obligate symbionts of many parasitic wasps in the families Ichneumonidae and Braconidae. They replicate only in specialized cells of the wasp ovary, the calyx cells, and are introduced into the host during oviposition (reviewed in Stoltz, 1993). In the parasitized host, they interfere with the host’s immune system to prevent encapsulation of the parasitoid egg and larva (reviewed in Lavine & Beckage, 1995) and in many cases they also affect host development (reviewed in Lawrence & Lanzrein, 1993; Stoltz, 1993). The genome of the polydnaviruses is segmented and consists of multiple circles of double-stranded DNA; viral DNA is integrated into the wasp’s genome and it is this proviral form of DNA that is responsible for the transmission of the viral genome from one generation to the next (reviewed in Fleming & Krell, 1993; Stoltz, 1993; Gruber et al., 1996; Savary et al., 1997).

In the parasitized host, polydnavirus DNA appears to persist without replication (Theilmann & Summers, 1986; Strand et al., 1992), and transcriptional activity has been documented in several hosts (reviewed in Stoltz, 1993; Strand et al., 1992; Harwood et al., 1994; Hayakawa et al., 1994; Yamanaka et al., 1996; Cui et al., 1997). In the case of the parasitoid/host system *Campoletis sonorensis/Heliothis virescens*, viral transcripts were also reported in the female parasitic wasp; viral gene expression was different in the two insect hosts, some transcripts being seen only in the parasitized host, others only in the female wasp and a third category in both hosts (Fleming et al., 1983; Theilmann & Summers, 1988). The temporal appearance of viral transcripts varies in the parasitized hosts investigated up to now. In *Heliothis virescens* parasitized by the ichneumonid *Campoletis sonorensis* and in *Pseudoplusia includens* parasitized by the braconid *Microplitis demolitor*, several viral transcripts were present from shortly after parasitization until the end of endoparasitoid development (Theilmann & Summers, 1988; Strand et al., 1992). On the
Other hand, in *Pieris rapae* parasitized by the braconid *Cotesia rubecula*, viral transcription appeared to be transient and was restricted to 4–8 h after parasitization (Asgari et al., 1996). These observations were all made with hosts of larval parasitoids, i.e. where the wasp oviposits into a larva, and it appears that the majority of transcripts analysed up to now are related to the strong immunosuppressive activity of the polydnaviruses in these systems (Dib-Hajj et al., 1993; Li & Webb, 1994; reviewed in Lavine & Beckage, 1995; Asgari et al., 1996; Cui et al., 1997).

Here, we present the first data on polydnavirus transcription in an egg–larval parasitoid and its host. In this type of parasitism, the wasp oviposits into eggs, a stage where immunocompetence appears to be lacking (Salt, 1968). The parasitoid larva then develops in the embryonic and larval stages of the host. We are working with the braconid *Chelonus inanitus* and its natural host, *Spodoptera litura*. This egg–larval parasitoid induces in its host the precocious onset of metamorphosis in the fifth instar and developmental arrest in the prepupal stage (Grossniklaus-Bürgin et al., 1994). Polydnavirus and venom have been shown to be responsible for the latter effect (Soller & Lanzrein, 1996) and to interfere with the endocrine system of the host in the last instar after pupal commitment (Grossniklaus-Bürgin et al., 1998). The presence of a parasitoid larva in addition to polydnavirus/venom was found to be necessary for induction of precocious metamorphosis and to interfere with the juvenile hormone system of the host (Pfister-Wilhelm & Lanzrein, 1996). Furthermore, polydnavirus/venom of *C. inanitus* have been shown to prevent encapsulation of the parasitoid larva (Pfister-Wilhelm & Lanzrein, 1996; Stettler et al., 1998), but this did not involve effects on haemocyte composition, ultrastructure, spreading behaviour or encapsulation activity (Stettler et al., 1998). We found that an elegant way to study the effect of polydnavirus/venom in the absence of the parasitoid larva is by X-ray irradiation of the female wasps (Soller & Lanzrein, 1996). Such wasps oviposit normally and inject polydnavirus/venom but the eggs are infertile and never develop. Host larvae developing from eggs that were parasitized with X-ray-irradiated wasps are hereafter referred to as X-ray-parasitized. Such larvae pass through six larval instars, as do non-parasitized larvae, but they become developmentally arrested in the prepupal stage, as do larvae developing from polydnavirus/venom-injected eggs (Soller & Lanzrein, 1996).

The baculovirus of *C. inanitus* (CiV) has been characterized and its genome was shown to consist of at least 10 different segments of between 7 and 31 kb (Albrecht et al., 1994). Several segments have been cloned and mapped (Albrecht et al., 1994) and integration into the wasp genome has been demonstrated for a 12 kb segment (CiV12) (Gruber et al., 1996). The excised circular DNA only appeared in females after a specific stage in pupal–adult development (Gruber et al., 1996). Here, we show by Northern dot blot analysis that very small amounts of viral transcripts were present in the early phase of parasitism, namely the egg and the early larval instars; transcript quantities increased in the penultimate and mainly the last instar. Some differences in the pattern of transcript abundance in the course of development were noted between the three segments tested, CiV12, CiV14 and CiV16 (Gruber et al., 1996). Based on sequence information, primers specific for three open reading frames (ORFs) were designed and used to perform RT–PCR. With this method, transcription of all three ORFs was documented in the parasitized host, the X-ray-parasitized host and also in wasp pupae.

### Methods

**Insects.** *S. littoralis* (Noctuidae, Lepidoptera) was mass-reared at 27 ± 1 °C with a photoperiod of 14 h and fed an artificial diet. Adult *S. littoralis* and diet were kindly given to us by Novartis AG, Basle. *C. inanitus* (Braconidae, Hymenoptera) is a solitary, egg–larval parasitoid and was reared on *S. littoralis*. Non-parasitized larvae pass through six larval instars, while parasitized larvae enter metamorphosis precociously in the fifth instar. The parasitoid larvae emerge from the developmentally arrested precocious prepupa. For details of the biology and rearing of the parasitoid and the host, see Grossniklaus-Bürgin et al. (1994). For experimental purposes, freshly laid eggs of *S. littoralis* were kept for 27–32 h at 20 °C before parasitization.

**X-ray irradiation of *C. inanitus* females.** X-ray irradiation of *C. inanitus* females (146 Gy ± 10%) was carried out as described previously (Soller & Lanzrein, 1996). The degree of parasitization was always verified by dissection of some of the parasitized eggs, and eggs and larvae were only used for experiments when parasitization was above 90%. Furthermore, some larvae were always kept for observation to verify the developmental arrest in the prepupal stage.

**Injection of calyx fluid/venom and implantation of *C. inanitus* larvae.** Calyx fluid and venom were collected from 1–2-day-old adult females of *C. inanitus* as described by Soller & Lanzrein (1996). Aliquots of 0·5–0·7 female equivalents of calyx fluid and venom, dissolved in 3 μl 0·1 M NaH2PO4/NaHPO4 with 7·5% (w/v) sucrose, pH 7·4, were injected into early feeding fifth instar larvae of *S. littoralis*. As a control, buffer alone was injected. Haemolymph was collected 24 h later and analysed for viral transcripts of CiV12 ORF2 (see below). In a separate experiment, 0·5–0·7 female equivalents of calyx fluid and venom, dissolved in 1 μl 0·1 M NaH2PO4/NaHPO4 with 7·5% (w/v) sucrose, pH 7·4, were injected into feeding fourth instar larvae of *S. littoralis* and 24 h later a late first instar female *C. inanitus* larva was implanted, i.e. when the recipients were young fifth instar larvae. The method of implantation of parasitoids was as described in Pfister-Wilhelm & Lanzrein (1996). Twenty-four hours after implantation of a parasitoid, the recipient was dissected and the condition of the parasitoid larva (survival, encapsulation) was checked.

**RNA isolation.** Parasitized, non-parasitized and X-ray-parasitized eggs or larvae of *S. littoralis* were frozen at −20 °C for at least 1 h or at most 1 week. Eggs or larvae (100 mg) were homogenized with a Polytron PT10-35/PTA 10s (Kinematica) in 0·45 ml lysis buffer RLT (RNeasy plant total RNA kit; Qiagen) plus 145 mM β-mercaptoethanol (10 μl per ml lysis buffer). The following steps were done according to the RNeasy plant mini protocol (Qiagen) with minor modifications. Briefly, the homogenate was incubated for 1–3 min at 56 °C, vortexed vigorously and applied to a QiShredder spin column and centrifuged for 2 min at 13000 g. The flow-through fractions were mixed with 0·5 vol. ethanol and then applied onto a RNeasy mini spin column and...
Centrifuged for 20–40 s at 7000 g. Next, a first DNA digestion was carried out: 10 µl DNase I (18 U/µl) in 90 µl DNase incubation buffer (High Pure isolation kit; Boehringer Mannheim) was pipetted onto the RNeasy membrane and incubated for 15 min at room temperature. Washing and elution was done according to the RNeasy plant mini protocol (Qiagen). As control PCR experiments with the RNA samples obtained by this procedure revealed that there was usually still some DNA present, a second DNA digestion was carried out with 4 µl DNase I (10 U/µl; Boehringer Mannheim) and 2 µl RNasin (40 U/µl; Promega) at room temperature for 50 min. The samples were then extracted once with 1 vol. acid phenol, once with 1 vol. acid phenol–chloroform–isoamyl alcohol (50:49:1) and once with 1 vol. chloroform–isoamyl alcohol (49:1). RNA precipitation was done in the presence of 0.5 vol. 3 M sodium acetate, pH 5.5, and 2.5 vols ice-cold ethanol at −20 °C overnight. The RNA pellets were dissolved in water and stored at −20 °C.

For isolation of RNA from haemolymph, parasitized fifth instar S. littoralis larvae were anaesthetized on ice and, after cutting away a caudal disk, haemolymph of three larvae was collected in 0.45 ml lysis buffer RLT plus β-mercaptoethanol. The samples were incubated for 1–3 min at 56 °C, vortexed vigorously and mixed directly with 0.5 vol. ethanol and then loaded onto the RNeasy mini spin column and processed as described above.

For isolation of RNA from pupae of C. ananassius, the method described by Chomczynski & Sacchi (1987) was used with some modifications. This RNA had been isolated before the above-described method was established. Briefly, two to five pupae from stages 1–6, as defined in Albrecht et al. (1994), were homogenized with a Polytron PT10-35/PTA 10s in approximately 500 µl 4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, and 100 mM β-mercaptoethanol, and 0.05 vol. 10% (w/v) Sarkosyl was then added. The procedure described by Chomczynski & Sacchi (1987) was then followed, but additional extractions with acid phenol and chloroform–isoamyl alcohol (49:1) were included. RNA was then precipitated with 0.1 vol. 3 M sodium acetate and 3 vols ice-cold ethanol for at least 2 h at −20 °C. After centrifugation, the pellet was dissolved in water and stored at −20 °C.

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DNA isolation. The method of Gruber et al. (1996) was used for isolation of DNA from calyx fluid or pupae of C. ananassius.

Results

To obtain an overview of CiV transcription in the course of parasitism and after parasitization with X-ray-irradiated wasps, 5 µg RNA from each developmental stage of parasitized and X-ray-parasitized hosts was probed with DIG-labelled CiV DNA or S. littoralis actin as an internal standard. As we knew that polydnavirus-induced developmental and endocrine

Polydnavirus transcripts in parasitoid and host
effects become manifest mainly after pupal commitment (Grossniklaus-Bürgin et al., 1998), we subdivided the last instar of X-ray-parasitized larvae into early and late feeding and early and late pupal cell formation stages. The Northern dot blots show that viral transcripts could be detected throughout the entire course of parasitization (Fig. 1a). While actin transcripts were found in similar quantities throughout the entire course of parasitism (Fig. 1b), quantities of viral transcripts increased towards the last instar, i.e. the fifth instar in truly parasitized larvae and the sixth instar in X-ray-parasitized larvae. Parasitized larvae become developmentally arrested in the fifth instar in the prepupal stage before the parasitoid larva emerges, and X-ray-parasitized larvae become developmentally arrested in the sixth instar at the prepupal stage. We never obtained a signal with the viral probe from RNA of non-parasitized eggs or non-parasitized larvae [Fig. 1(a) and data not shown], whereas a strong signal was seen with the actin probe (Fig. 1b). RNA from haemolymph of fifth instar parasitized larvae gave a clear signal with the viral probe (Fig. 1a), indicating the presence of viral transcripts in haemocytes. In general, the viral transcripts only became visible after 5–20-fold longer exposures than the actin transcripts, which indicates that only very small amounts of viral transcripts were present, particularly in the early phase of parasitism.

We then compared transcription from three viral segments in the course of development in parasitized and X-ray-parasitized hosts. RNA (5 µg) from eggs and each larval instar was hybridized with DIG-labelled segments CiV12, CiV14 and CiV16-8 (Fig. 2). As it is not possible to probe the same blot sequentially at high sensitivity with the DIG system, separate blots were made with the same RNA. CiV12 transcripts were seen in all stages investigated and the greatest quantities were seen in late feeding fifth instar parasitized larvae and in X-ray-parasitized larvae at the late feeding and early cell formation stages (Fig. 2a). CiV14 transcripts were also seen throughout parasitization, with the greatest quantities in late feeding fifth instar parasitized larvae and in X-ray-parasitized larvae at the late feeding and early cell formation stages (Fig. 2a). CiV14 transcripts were also seen throughout parasitization, with the greatest quantities in late feeding fifth instar parasitized larvae and in X-ray-parasitized larvae at the late feeding and early cell formation stages (Fig. 2a). CiV16-8 transcripts were also found throughout parasitization but, in contrast to CiV12 and CiV14, the quantity had already increased in the penultimate instar and decreased at the cell formation stage (Fig. 2c). In haemolymph, rather large quantities of transcripts were seen with all three segments. These observations were confirmed in other independent experiments.
Having found viral transcripts of all three segments tested throughout parasitization, we then focused our attention on the analysis, by RT–PCR, of two ORFs on CiV12 and one on CiV16–8. ORF sequence comparisons did not reveal any information regarding function. The same RNA as was used in the Northern dot blots was used for the RT–PCR experiments. Products of the expected length (262 bp) could be found for CiV12 ORF2 in all larval instars of the parasitized host (Fig. 3a) and also in all larval instars of X-ray-parasitized hosts until developmental arrest; no product was found in the stationary prepupae (Fig. 3b). Also, the same PCR product was formed with RNA from eggs and haemolymph of parasitized and X-ray-parasitized larvae, whereas no PCR product was formed with RNA from non-parasitized eggs or larvae (data not shown). We next investigated the transcription of CiV12 ORF2 in female and male wasps over the course of pupal–adult development. A product of the expected length was formed in both females (Fig. 4a) and males (Fig. 4b); in the former, the signal was stronger in stages P3b to P6, i.e. when viral DNA is amplified and virions are formed (Albrecht et al., 1994; Gruber et al., 1996). We next analysed transcription of CiV12 ORF1 and CiV16–8 ORF1 in a similar manner. For both ORFs, a product of the expected length (140 bp for CiV12 ORF1 and 238 bp for CiV16–8 ORF1) was found in parasitized and X-ray-parasitized fifth instar larvae, haemolymph of fifth instar parasitized larvae and also female wasp pupae stage P4, but not in non-parasitized larvae (Fig. 5).

We next investigated whether viral DNA was transcribed when virions were injected manually into S. littoralis larvae and whether this could protect an implanted parasitoid larva from encapsulation. We thus injected calyx fluid/venom into young fifth instar larvae and analysed their haemolymph for transcription of CiV12 ORF2 by RT–PCR. Fig. 6 shows that a product of the expected length was formed in haemolymph of S. littoralis larvae 24 h after injection of calyx fluid/venom; as control experiments showed that calyx fluid contained no viral RNA, this indicates that viral DNA is transcribed when virions are injected into S. littoralis larvae, which is an unnatural developmental stage for virus entry for an egg–larval
parasitoid. To investigate whether injection of calyx fluid/venom might prevent encapsulation of implanted parasitoids, 12 parasitoids were implanted individually into 12 S. littoralis larvae that had been injected 24 h earlier with calyx fluid/venom. Dissection of the recipient larvae 24 h after implantation revealed that all parasitoids were encapsulated. This shows that the injected virions were not able to protect the parasitoid larva, despite the fact that viral transcripts were formed.

Discussion

The Northern dot blot analyses with viral DNA (Figs 1a and 2) or, as an internal standard, actin DNA (Fig. 1b) as probe revealed the presence of only very small quantities of viral transcripts in the early phase of parasitism. Quantities of viral transcripts were also initially small in X-ray-parasitized eggs and larvae, which contain polydnavirus and venom but no parasitoid larva (Soller & Lanzrein, 1996). Larger quantities of viral transcripts were found mainly in the last larval instar, i.e. the fifth instar in truly parasitized and the sixth instar in X-ray-parasitized larvae. Thus, the parasitoid larva caused a precocious increase in viral transcripts, along with the precocious onset of metamorphosis. It is in the last instar that the effects of parasitization (Grossniklaus-Bürgin et al., 1994) or X-ray-parasitization (Grossniklaus-Bürgin et al., 1998) on host development become clearly manifest, and the increase in viral transcripts in the last instar thus appears to be associated to some extent with the polydnavirus/venom-induced developmental arrest. The increase in viral transcripts towards the later phase of parasitization is even more pronounced if considered on a per-host basis: the 5 µg RNA blotted per dot corresponds to approximately 20–30 eggs, 7–8 first instar larvae, 3–5 second and third instar larvae and 0.5–1 fourth to sixth instar larvae. As viral DNA does not replicate in the parasitized host, this means that CiV is almost silent transcriptionally during the initial 7–8 days after parasitization and only becomes active in the last few days before developmental arrest and parasitoid emergence. These are the first data on viral transcript levels in the host of an egg–larval parasitoid and they are totally different from those reported for larval parasitoids, where large quantities of transcripts were seen only shortly after parasitization (Asgari et al., 1996) or from shortly after parasitization until parasitoid emergence (Theilmann & Summers, 1988; Strand et al., 1992). As protection of the parasitoid egg from encapsulation is a major function of polydnaviruses in larval parasitoids, the majority of viral transcripts of larval parasitoids described up to now appear to be related to the immunosuppressive function of the polydnaviruses (Dib-Hajj et al., 1993; Li & Webb, 1994; Asgari et al., 1996).

Viral transcription was seen to be slightly different for the three segments tested (Fig. 2). CiV16.8 transcripts had already increased in the penultimate larval instar, i.e. the fourth in parasitized and the fifth in X-ray-parasitized larvae, whereas CiV12 and CiV14 transcripts increased in the last larval instar, i.e. the fifth in parasitized larvae and the sixth in X-ray-parasitized larvae. In X-ray-parasitized larvae, where no parasitoid larva develops and emerges, various endocrine parameters have been analysed in the phases of the last instar that precede the polydnavirus/venom-induced developmental arrest. Ecdysone release by prothoracic glands was suppressed in the early cell formation stage in X-ray-parasitized larvae and juvenile hormone and juvenile hormone esterase concentrations were reduced in the late cell formation and early prepupal stages when compared with non-parasitized larvae (Grossniklaus-Bürgin et al., 1998). As CiV14 transcript levels were highest from the late feeding to the late cell formation stage and CiV12 transcript quantities from the late feeding to the early cell formation stage, some of the transcripts of these two viral segments might be involved in causing these endocrine effects.

The Northern dot blots with RNA from haemolymph always gave a strong signal relative to that with RNA from whole body extracts with either calyx DNA or the three viral segments tested as the probe (Figs 1 and 2). At present, we do not know the function of these viral transcripts but we know that polydnavirus/venom protect the parasitoid larva from encapsulation without affecting differential haemocyte count, haemocyte morphology, ultrastructure or encapsulation activity (Stettler et al., 1998). Transcript levels were also high in haemocytes from hosts of larval parasitoids (Strand et al., 1992; Hayakawa et al., 1994; Lavine & Beckage, 1995; Yamanaka et al., 1996); polydnaviruses affect haemocyte spreading and encapsulation activity in larval parasitoids and in some cases also affect haemocyte morphology and ultrastructure (reviewed in Stettler et al., 1998).

The experiment where polydnavirus transcription was observed after calyx fluid/venom injection into larvae (Fig. 6) shows that virus transcription takes place even when the virus
enters the host at an unnatural developmental stage, i.e. when it is injected into a larva instead of an egg. The injected calyx fluid/venom failed to protect the implanted parasitoid larva from encapsulation, whereas injection of calyx fluid was seen to protect the encapsulation of injected eggs in several larval parasitoids (reviewed in Stettler et al., 1998). The effect of polydnavirus/venom of *Chelonus inanitus* on host development, on the other hand, can be partially mimicked by injection of calyx fluid/venom into third and fourth instar larvae of *S. littoralis* as these larvae become prepupal–pupal intermediates (R. Pfister-Wilhelm, personal communication).

The analyses of three ORFs by RT–PCR revealed the absence of host specificity, as transcripts were found in parasitized and X-ray-parasitized *S. littoralis* as well as in female pupae of *C. inanitus* (Figs 3–5). Transcription of CiV12 ORF2 appeared to increase in parallel with the replication of viral DNA in female pupae (Gruber et al., 1996) and transcription was also documented for male pupae (Fig. 4). This is the first demonstration of polydnavirus transcription in male parasitic wasps. Since no circular viral DNA appears to exist in male *C. inanitus* pupae or in stage 1 and 2 female pupae (Gruber et al., 1996), the CiV12 ORF2 transcript seen at these stages seems to stem from the chromosomally integrated form of viral DNA. In *Campoletis sonorensis*, cDNA probes synthesized from poly(A)+ mRNA from adult male or female wasps did not hybridize with viral DNA to detectable levels, whereas probes from pupae (males and females mixed) hybridized with several viral fragments (Fleming et al., 1983). However, one has to keep in mind that RT–PCR is a very sensitive method, and it is conceivable that more viral transcripts would be found in other parasitoids and their hosts by RT–PCR than have currently been described, where up to now Northern analyses have always been used.

In conclusion, we have documented polydnavirus transcription in an egg–larval parasitoid and its host for the first time and showed an increase in viral transcripts towards the final phase of parasitization, when developmental alterations become manifest. At present, we do not know whether the transcripts are polyadenylated and become translated, nor do we know the function of the encoded proteins. Screening of cDNA libraries of relevant developmental stages of the host with viral segments and fragments thereof will be a next step in trying to elucidate the role of polydnavirus genes in affecting host development.

P.S. did the initial work on this subject and A.J. and P.S. contributed equally to the development of the project. We would like to express our thanks to Novartis AG, Basle, for providing us with adult *S. littoralis* and the diet for rearing the larvae and to Dr D. Gerling, Tel Aviv, for collecting the progenitors of our *C. inanitus* colony for us. We thank Rita Pfister-Wilhelm for contributions to parasitoid rearing and X-ray irradiation of wasps and for having made the calyx fluid/venom injections as well as the parasitoid implantations. We also thank Professors Daniel Schümperli and Isabel Roditi for helpful discussions and technical advice, Toni Wyler for photographic work and Dr P. Schneeberger (Radiology, University Hospital, Berne) for allowing us to use the X-ray irradiation instrument. Financial support from the Swiss National Science Foundation (grants 31–41828.94 and 31–52399.97) to B.L. is also gratefully acknowledged.

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Received 4 March 1999; Accepted 31 March 1999