

Short Communication

Establishment of cell lines from the wasp *Hyposoter didymator* (Hym., Ichneumonidae) containing the symbiotic polydnavirus *H. didymator* ichnovirus

Janick Rocher, Marc Ravallec, Patrick Barry, Anne-Nathalie Volkoff, Dominique Ray, Gérard Devauchelle and Martine Duonor-Cérutti

Laboratoire de Pathologie Comparée, INRA, 30380 Saint Christol-lez-Alès, France

Correspondence
Anne-Nathalie Volkoff
volkoff@ensam.inra.fr

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Cell lines derived from polydnavirus-associated wasps should constitute a valuable tool for investigations of polydnavirus replication, but none is yet available. In this work, we describe the first cell lines, named Hd-AA, -AD, -BBA and -K, to have been established from the ichneumonid wasp *Hyposoter didymator*, associated with the polydnavirus *H. didymator* ichnovirus (HdIV). Southern blot analysis indicated that the viral DNA was present in all four cell lines and co-localized with high molecular mass DNA, probably the wasp chromosomes. Northern blot analysis of mRNAs extracted from the AA cell line showed transcription of some HdIV-encoded genes, although at low level. The effects of ecdysone treatment, HdIV re-infection and 42 °C heat-shock were analysed in the AA cell line. No effect was detected at the DNA (virus replication) or RNA (gene expression) levels, which may be due to the limitation of the present available tools.

Polydnaviruses are symbiotic viruses associated with some parasitic wasps from the ichneumonid and braconid families. They are maintained in the wasp population by stable integration of proviral DNA in the wasp genome (Stoltz *et al.*, 1986; Fleming & Summers, 1991). Unique among the viruses, they require two distinct insect hosts to achieve their complete life-cycle. Replication occurs solely in the calyx epithelial cells of the female wasp oviduct leading to an encapsidated viral genome composed of several circular molecules of double-stranded DNA in a non-equimolar ratio. The viral particles accumulate in the female oviducts and are injected during parasitization into a lepidopteran host along with the parasitoid egg. In the lepidoptera, infection of different tissues leads to expression of a host-specific subset of viral genes. Viral gene expression is mandatory for parasitoid survival and development by inducing physiological alterations in the lepidopteran host (reviewed by Webb, 1998).

During replication, viral DNA is excised from the wasp genome then packaged into virions. Recent data obtained in braconids suggest that amplification of viral DNA precedes excision and circularization (Marti *et al.*, 2003; Pasquier-Barre *et al.*, 2002). The mechanisms of polydnavirus replication are as yet unknown and represent a puzzling mystery for virologists. Replication is tissue-specific and development-dependent as it occurs solely in specialized cells of the female wasp oviduct and is first detected at the end of pupal development (Norton & Vinson, 1983; Volkoff *et al.*, 1995). The regulation of virus replication is probably

related to the calyx cell differentiation and is thus indirectly related to the biologically active derivatives of the insect hormone ecdysone, 20-hydroxyecdysone (20HE) (Marti *et al.*, 2003; Webb & Summers, 1992). In addressing the question of polydnavirus replication, researchers have to date been limited by the impossibility of promoting *in vitro* replication and a lack of appropriate tools (Stoltz, 1993). In this work, we describe cell lines that were established from *Hyposoter didymator* pupae. *H. didymator* is an ichneumonid wasp associated with the polydnavirus *H. didymator* ichnovirus (HdIV). These cell lines are, to our knowledge, the first to be derived from a polydnavirus-associated wasp and may constitute a valuable tool for future investigations on polydnavirus replication.

The cell lines were established from young *H. didymator* pupae (0–24 h after meconium release). Cocoons were washed in 20% bleach solution, then rinsed twice with sterile water. Pupae of both sexes were recovered and washed rapidly in 5% bleach, then rinsed twice with sterile water. The dissected pupae were stored in HdM cell culture medium until a sufficient amount of material had been collected (20–25 pupae). HdM is composed of TC100 modified medium (Gibco) supplemented with 20% foetal calf serum (Gibco), 1% G5 complement (Gibco), 4% *Kniphofia* nectar (Pronectar SA), 1.61 g putrescine l⁻¹ (Sigma), 50 units nystatin ml⁻¹ (Sigma) and 1.25 µg fungizone ml⁻¹ (Gibco). Pupae were then placed in a Petri dish, rinsed with sterile PBS, and cut into small pieces with two scalpel blades. The lacerated samples were dissociated

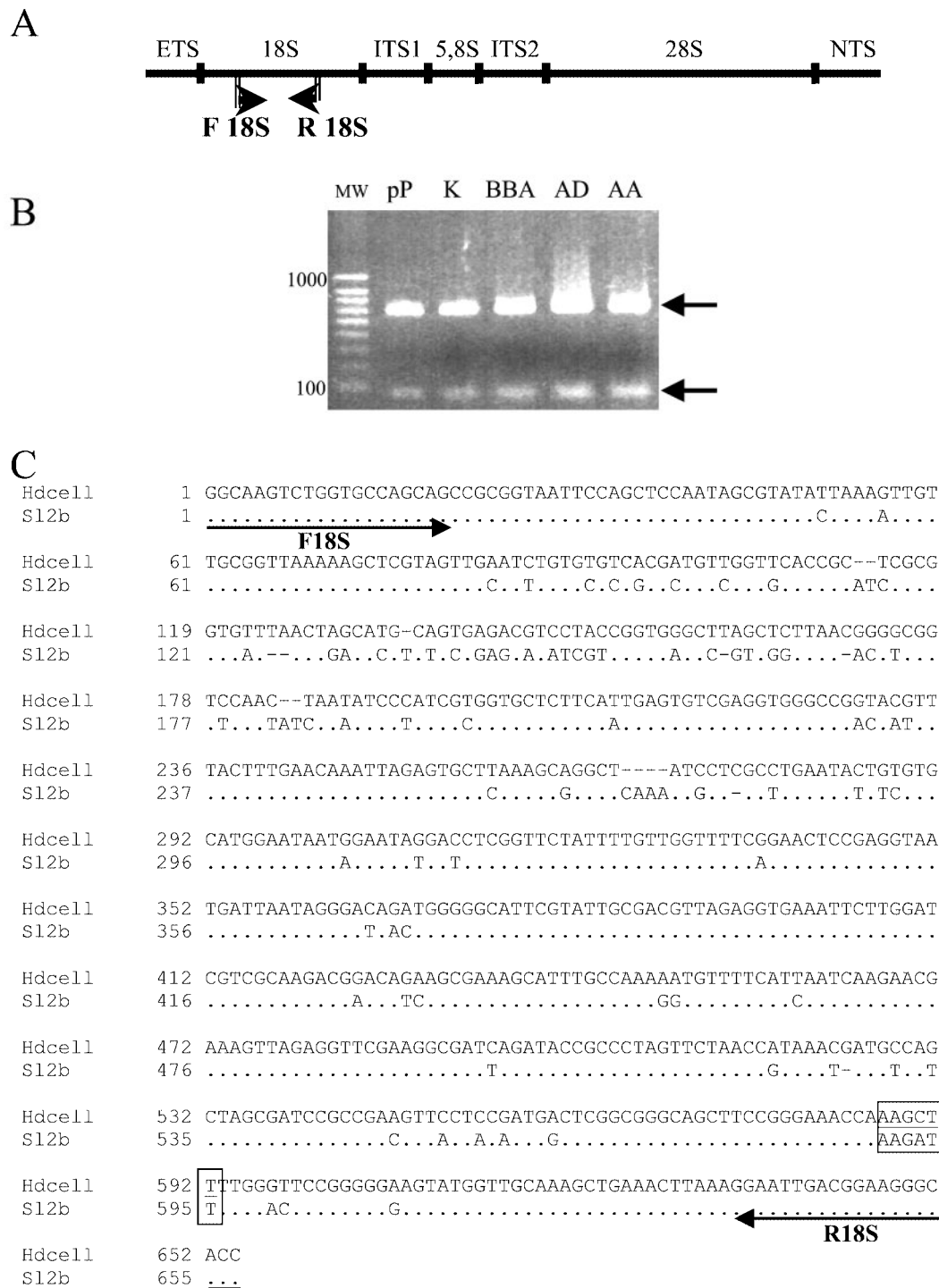


Fig. 1. Control of wasp origin of the established cell lines. (A) Position of the primers F18S and R18S in conserved regions of the 18S rDNA used for PCR amplification. (B) *Hind*III digest of the obtained PCR products, after separation by agarose gel electrophoresis and ethidium bromide staining. Molecular masses are indicated on the left. Templates were *H. didymator* pre-pupae (pP) and the Hd cells (AD, AA, K and BBA). Arrows indicate the two restriction fragments. (C) ClustalX alignment of the sequences of the PCR products obtained from the Hd-AA cell line (Hdcell; GenBank AY433942) and the S12b cell line (C. Pelle, S. Gimenez & P. Fournier, unpublished) showing the nucleotides that differ between the two sequences. Primer sequences are indicated by arrows and the region containing the hymenoptera-specific *Hind*III restriction site is boxed.

either by incubation in trypsin/EDTA solution (2.0 mg trypsin ml⁻¹, 0.08 M EDTA) for 1 min or by incubation in collagenase solution (0.5 mg ml⁻¹) for 2 min. Samples were washed twice by centrifugation (5 min, 1000 r.p.m.), resuspended in 3 ml HdM and placed in a 12.5 cm² flask (Falcon) at 28 °C. The culture medium was replaced by fresh HdM or HdM^{0.5} (containing 0.5% G5 complement) the following day and then periodically until establishment of the cell lines. After about 2 years, four different cell lines, named Hd-AA, -AD, -BBA and -K, were established. The AA and AD cell lines were established following incubation in trypsin/EDTA solution. AA cells were kept in HdM^{0.5}, while AD cells were maintained in HdM. The BBA and K cell lines were established following incubation in collagenase solution. BBA cells were kept in HdM, whereas K cells were maintained in HdM^{0.5}.

To control the wasp origin of the cell lines and discard the possible risk of contamination by the lepidopteran host *Spodoptera littoralis* cells, 18S rDNA sequences from the Hd cells, *H. didymator* pre-pupae and *S. littoralis* cells and eggs were PCR amplified and analysed. Primers F18SHd (5'-GACTGACGTCGGCAAGTCTGGTGCCAGCAG-3') and R18SHd (5'-CATGGAATTCGGTGCCCTTCCGTCAATCC-3') were designed in conserved regions flanking a sequence shown as variable after alignment of sequences deposited in GenBank for lepidopteran and hymenopteran species (Fig. 1A). PCR amplification was conducted using *Taq* DNA polymerase (Roche) with 100 ng template DNA extracted from the lepidopteran cell lines Sf9 and Sl2b (*S. littoralis* haemocyte cell line; Volkoff *et al.*, 1999) and from the wasp cell lines AA (15th passage), BBA (19th passage), AD (19th passage) and K (20th passage) according to a standard protocol (Ausubel *et al.*, 1995). DNA was also extracted from wasp pre-pupae and *S. littoralis* eggs. According to sequences available in GenBank from other insects, a *Hind*III restriction site was predicted to be conserved in the amplified hymenopteran sequences, but absent in the lepidopteran ones. As predicted, all the amplification products from *H. didymator* cells and insects were digested by this enzyme (Fig. 1B) but none from *Spodoptera* (data not shown). The wasp origin of the established cell lines was confirmed by sequencing of the PCR products. Indeed, rDNA from the four cell lines was found to be different from the *S. littoralis* sequence (Fig. 1C) and identical to the wasp pupae rDNA (data not shown).

The four Hd cell lines could be differentiated by their general morphology (Fig. 2), suggesting they differed with regard to their genetic/phenotypic background. Cells were observed by light and electron microscopy. For electron microscopy, cells were pelleted by centrifugation (5000 g, 10 min), then fixed and observed as previously described (Volkoff *et al.*, 1995). The AA and K cells were isolated cells but tended to form aggregates. The AA cell population was mainly composed of fusiform cells, whereas the K cells were star-shaped cells in contact with each other via well-developed pseudopodia. Their doubling time was,

respectively, 2 and 4 days. The two other cell lines, AD and BBA, showed more evident intercellular junctions. The BBA cells were small round cells that grew very slowly (doubling time of 8 days). The AD cells (with a doubling time of 4 days) were characterized by a large nucleus. The latter were strongly adherent to the substrate and grew in a cohesive monolayer. Interestingly, early after establishment (8th passage), the AD cell line, when submitted to 20HE treatment, formed organized tissue-like structures (Fig. 2). However, 3 years later (~107th passage), 20HE treatment induced no more differences in cell morphology.

For karyotype observations, cells were seeded for 24 h on to microscope coverslips and treated with 1.5 µg colcemid ml⁻¹ for 7 h. Fixation, DAPI staining and observations were performed as previously described (Gerbal *et al.*, 2000). In the Hd cells, the chromosome number was significantly higher than that found in the haplo-diploid insect ($n=12$, data not shown). It generally varied from $3n$ to $5n$, depending on the cell line (Fig. 2); the most variable population was found in the AA cell line, where some cells contained up to $10n$ chromosomes. This increase in chromosome number linked to cell line establishment was not surprising and has been previously reported for other cell lines (Gerbal *et al.*, 2000).

As the HdIV genome was predicted to be integrated in the wasp chromosomes, Southern blot analysis was performed to analyse the HdIV DNA status in the four cell lines. The procedure employed for hybridization was as described by Volkoff *et al.* (1999). The probe consisted of [α -³²P]dCTP-labelled (Nick-translation kit; Roche) total viral DNA. The results indicated that the HdIV DNA probe hybridized with the AA, AD, BBA and K cell genomic DNA, while no hybridization was observed with the lepidopteran cell samples (Fig. 3A). No circular viral DNA, as observed in encapsidated virus (Fig. 3A, lane HdIV), could be detected in the cell lines. The hybridization signal co-localized with high molecular mass DNA, suggesting integration of the viral DNA into the wasp cell chromosomes.

Interestingly, some viral transcripts were detected in the AA cells when mRNA was probed with total HdIV DNA, but at very low level compared with HdIV-infected Sf9 cells (Fig. 3B, compare lanes C and Sf+V). We do not know if the low level of gene expression observed in the AA cells has a biological significance. It could be related to the existence of low amounts of excised DNA in the wasp cells, which has been described in non-calyx cells of other wasps (Savary *et al.*, 1999), despite the fact that we could not detect this by Southern blotting (Fig. 3 A and D). In the wasp, the virus particles stored in the oviduct have never been reported to infect the insect cells, which could be due to a mechanical barrier or another unknown mechanism. Nevertheless, HdIV particles may enter wasp cell lines (Fig. 3C) when the latter are infected *in vitro* [infections were conducted as described by Volkoff *et al.* (1999) using 40 *H. didymator* females per ~10⁶ cells for 4 h]. When transcripts were analysed in the infected AA cell

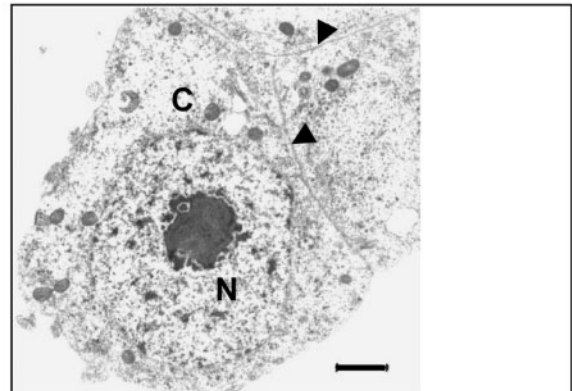
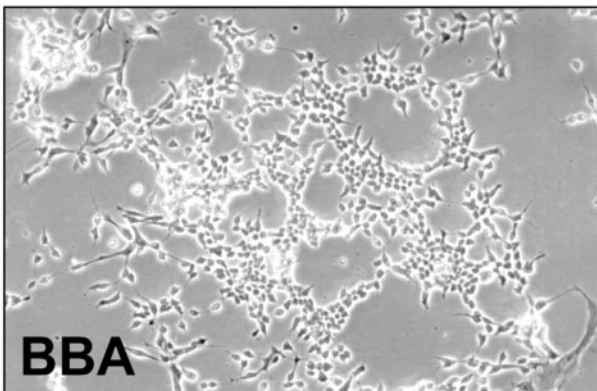
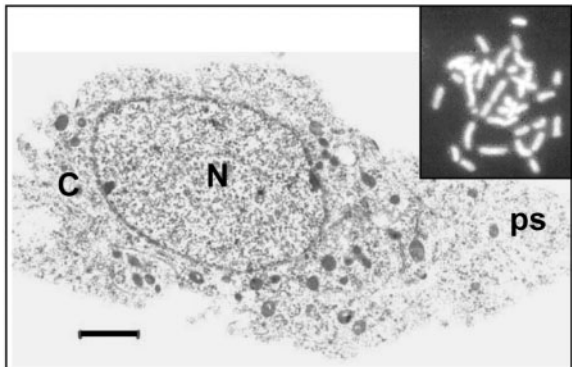
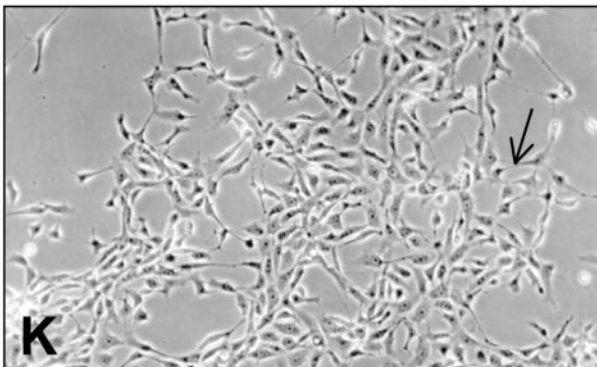
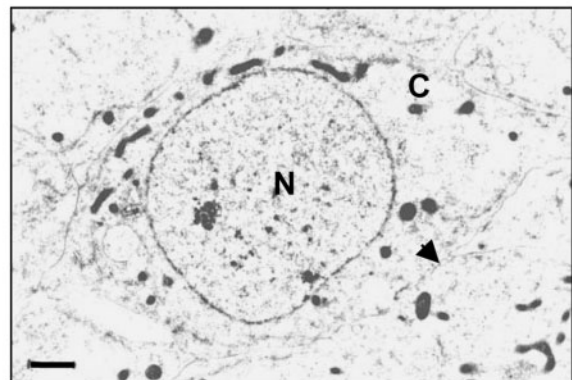
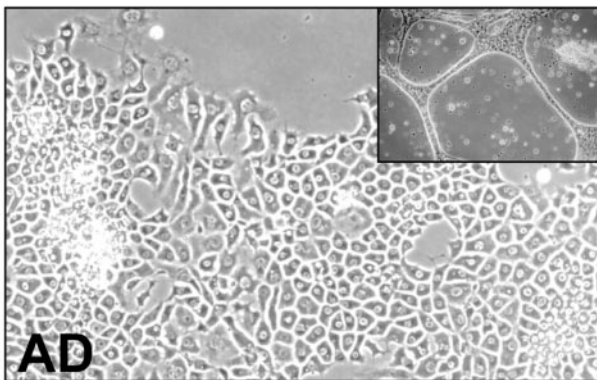
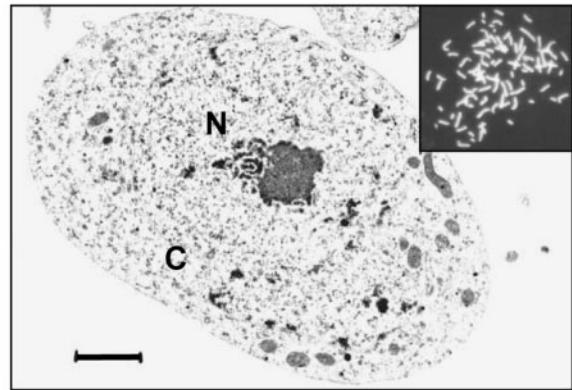
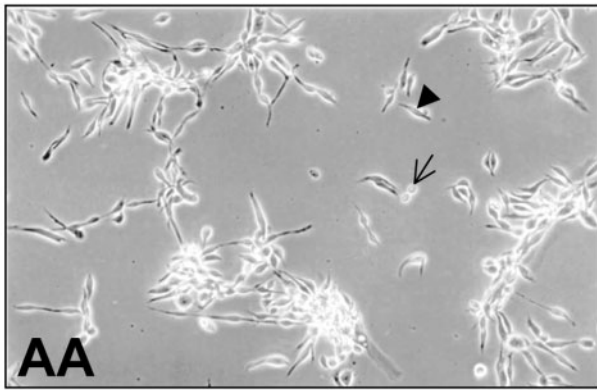


Fig. 2. Morphology of the *Hyposoter didymator* cell lines AA, AD, K and BBA. Left panels shows phase-contrast observations. AA panel: the arrowhead indicates a fusiform cell; the arrow indicates two spherical cells. K panel: arrow indicates a pseudopodium connecting two cells. AD panel, insert: tissue-like structure observed by phase-contrast microscopy 48 h after 20HE treatment (10^{-7} mg ml $^{-1}$). Right panels show transmission electron microscopy views. The nucleus (N) and cytoplasm (C) are indicated. In the AD and BBA panels, arrowheads indicate cell–cell junctions; in the K panel, ps indicates a pseudopodium. Bar, 1 μ m. In the AA and K cell panels, DAPI-stained chromosomes are shown indicating the polyploidization of the cells in culture ($>5n$ for AA cells and $\sim 3n$ for K cells).

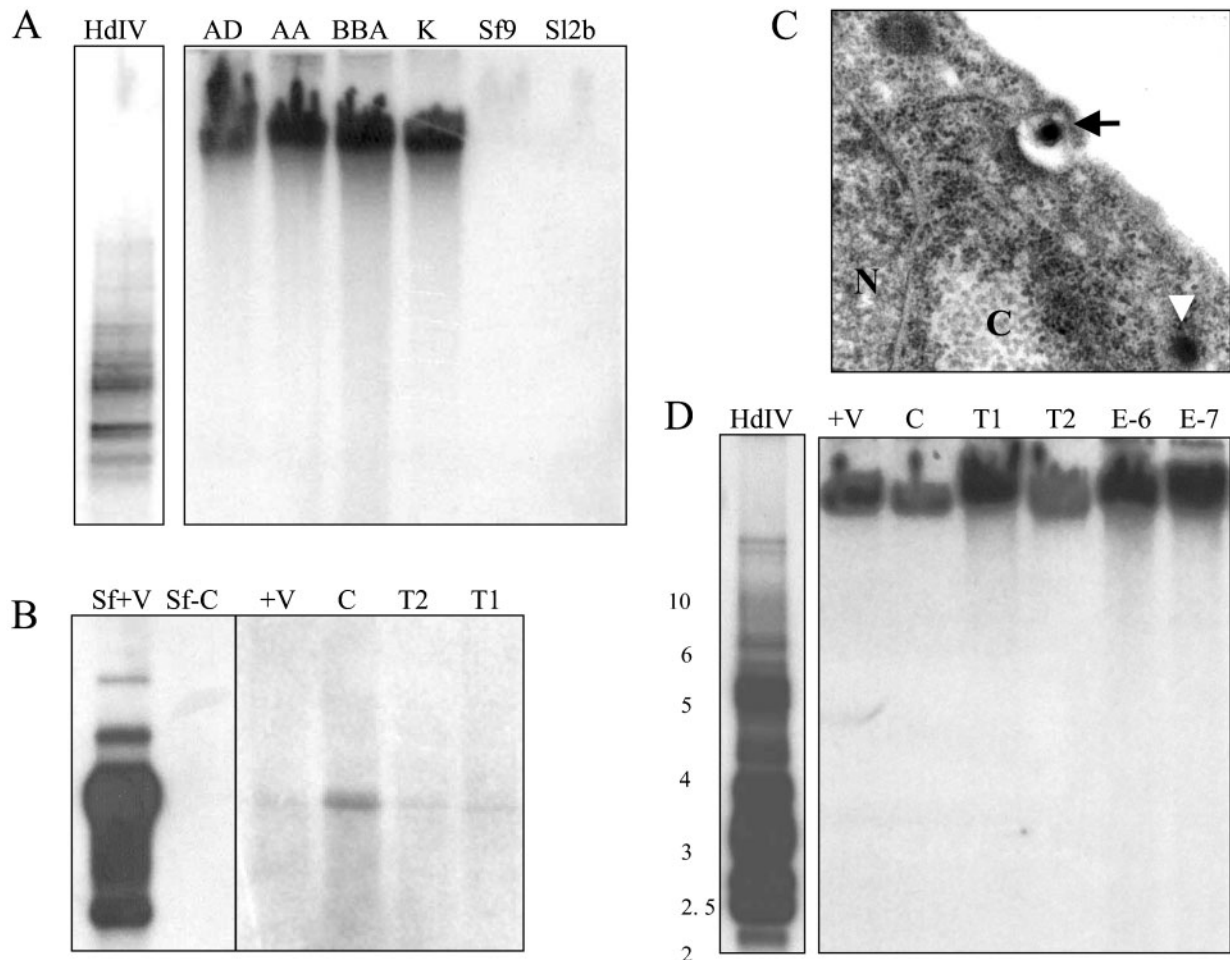


Fig. 3. Polydnviral DNA and RNA status in the wasp cell lines. (A) Southern blot analysis of the Hd cell lines. DNA was extracted from the lepidopteran cell lines Sf9 and SI2b and from the wasp cell lines AA (80th passage), BBA (51st passage), AD (78th passage) and K (38th passage) according to a standard protocol (Ausubel *et al.*, 1995). Approximately 20 μ g of each sample and 5 μ g of control viral DNA (HdIV) were separated by electrophoresis on a 0.8% TAE agarose gel. (B) Northern blot analysis of the AA cell line. Total RNA was extracted from uninfected and HdIV-infected Sf9 (Sf-C and Sf+V, respectively) and AA cells (C and +V, respectively) and from 42 °C heat-shocked AA cells (101st passage). *In vitro* cell infections (+V) were conducted for 4 h and samples were analysed 40 h post-infection. Heat-shocked cells were treated for 1 or 2 h (T1 and T2, respectively) and analysed 24 h after treatment. Approximately 15 μ g mRNA was loaded per sample. The film was exposed for 48 h for lanes Sf+V and Sf-C and for 15 days for the others. (C) HdIV infection of K cells observed by transmission electron microscopy: an HdIV particle is entering the cell by endocytosis (arrow); another (arrowhead) is in the cell cytoplasm (C); N indicates the cell nucleus. (D) Southern blot analysis of the AA cell line. DNAs were extracted from uninfected (C) and HdIV-infected (+V), 42 °C heat-shocked and 20HE-treated AA cells (106th passage). *In vitro* cell infection and heat-shock treatment were as described in (B). Samples E-6 and E-7 were treated with two concentrations of 20-hydroxyecdysone [10^{-6} and 10^{-7} mg (ml solution) $^{-1}$; respectively] for 48 h and analysed 24 h after treatment. Electrophoresis was carried out as in (A). For all blot analysis, the probe consisted of [α - 32 P]dCTP-labelled total HdIV DNA. Molecular masses (kbp) are indicated on the left.

lines by Northern blotting, using labelled HdIV DNA as a probe, no difference was found compared with non-infected cells (Fig. 3B, compare lanes +V and C). This may indicate that only a small number of HdIV particles infect Hd cells or that Hd cells lack factors allowing virus expression, as is the case with lepidopteran cells. When DNA status was analysed in HdIV-infected AA cells, no circular viral DNA could be detected (Fig. 3D, lane +V), suggesting the absence of excision/circularization of viral DNA in these cells. A quantitative analysis will nevertheless be necessary to determine whether DNA amplification is taking place within the wasp chromosomes.

Since 20HE is known to be related to polydnavirus replication, the AA cell line was submitted to increasing concentrations of 20HE and analysed by Southern and Northern blotting. Treatment was found to be deleterious, with observation of cell lysis, when doses were greater than 10^{-6} mg ml⁻¹. At lower doses, 20HE treatment did not affect HdIV DNA status in AA cells (Fig. 3D, lanes E-6 and E-7) and did not promote expression of detectable HdIV genes (data not shown). A 42 °C heat-shock was also performed to attempt to trigger cellular pathways leading to virus replication. Cells were submitted to heat-shock by placing the culture flask in a 42 °C water-bath for 1 or 2 h. AA cells responded to heat-shock since a wasp hsp90 homologue transcript was detected in the samples using a *S. frugiperda* hsp90 probe (Landais *et al.*, 2001) (data not shown), but no effect was observed on HdIV transcription (Fig. 3B, lanes T1 and T2) nor on viral DNA status (Fig. 3D, lanes T1 and T2).

Thus, neither 20HE treatment, HdIV infection, nor 42 °C heat-shock applied to the AA cell line promoted virus replication, at least at a level detectable with the currently available tools, in the wasp cells in culture. It should be noted that sequencing data now available on several polydnavirus genomes indicate that encapsidated genomes encode few genes related to virus replication (Turnbull & Webb, 2002). Therefore, because the probe used consisted of the HdIV encapsidated genome, we cannot exclude the possibility that other genes (non-encapsidated viral genes, if they exist, or wasp cellular genes) that may be involved in virus replication are transcribed but not detected in the AA cells. Furthermore, as mentioned by Webb & Summers (1992), there are probably many factors involved in the regulation of polydnavirus replication, such as sex, developmental and tissue-specific factors, that are yet to be identified.

To conclude, the *H. didymator* cell lines described in this work are some of the few established from hymenoptera insects. They contain a polydnavirus genome and should be a valuable tool to study the status of polydnavirus genomes in their wasp hosts and to analyse various factors that may lead to HdIV replication. Having these cell lines available thus offers great potential to understand better the still mysterious polydnavirus organization and life-cycle.

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