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# Insecticidal activity of a recombinant baculovirus containing an antisense c-*myc* fragment

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**Attempts to develop baculovirus-based insecticides by insertion of genes encoding enzyme inhibitors, neuropeptides or toxins have met with some success. However, it is often difficult to ensure correct processing or secretion of the encoded peptides. Here we tested a simpler strategy by insertion of an antisense fragment of a host gene to block translation of a protein essential for larval growth and development. We selected the c-*myc* gene for two main reasons: (i) its protein is known to be well conserved in evolution and to have multiple essential functions during development; and (ii) c-*myc* family genes have yet to be characterized in insects, thus blockage of essential genes by antisense transcripts from a strong virus promoter could provide a sensitive test for the existence of *myc*-like gene products. An appropriate fragment of the human c-*myc* gene was inserted downstream from the polyhedrin promoter of *Autographa californica* nucleopolyhedrovirus and tested in bioassays on *Spodoptera frugiperda* larvae. Western blot analysis with a human c-*myc* antibody revealed an endogenous protein band which bound specifically to these antibodies. This band disappeared more rapidly from cells infected with the antisense c-*myc* recombinant virus than from those infected with c-*myc*-negative virus. Results of bioassays showed that the antisense construct stopped feeding as soon as the polyhedrin promoter-driven transcripts accumulated, followed shortly by death of the larvae. These results suggest that c-*myc*-like protein(s) exist in insects and that the antisense strategy is an effective approach to virus insecticide production.**

## Introduction

The development of biopesticides as a viable alternative to chemical insecticides has suffered from a number of drawbacks, mostly due to their lower efficacy and higher costs. Yet safety concerns dictate that biological control agents must be sought in the future to provide target-specific insecticidal activity. Among the known insect pathogens, baculoviruses appear particularly attractive for two main reasons: (i) they are naturally involved in the regulation of insect populations including many agricultural and forest pests; and (ii) they are found solely in arthropods and individual strains have a very narrow host range and are, therefore, harmless to non-target insects and other organisms (reviewed by Groner, 1986). Baculoviruses are ideal agents for biological control when one considers environmental quality and safety but from the viewpoint of crop protection they are usually inefficient. Baculovirus-infected larvae continue feeding for long periods of time, inflicting nearly as much damage as uninfected insects (Hunter *et al.*, 1984). One strategy, therefore, has been to develop viruses which immobilize and kill their insect hosts more quickly.

Success in this direction has been achieved through the introduction of genes encoding bacterial or arthropod insect-selective toxins or juvenile hormone inhibitors (Carbonell *et al.*, 1988; Merryweather *et al.*; 1990; Tomalski & Miller, 1991; Stewart *et al.*, 1991; Maeda *et al.*, 1991; Tomalski *et al.*, 1993; Ribeiro & Crook, 1993; Cory *et al.*, 1994; Chejanovsky *et al.*, 1995; Eldridge *et al.*, 1995; Heinz *et al.*, 1995). However, concerns remain over target selectivity and the possibility of development of insect resistance to the foreign protein, as illustrated in the case of the *Bacillus thuringiensis* toxin (McGaughey, 1994). With this in mind, we opted for an

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alternate strategy: the use of a strong virus promoter to produce an excess of antisense transcripts complementary to the mRNA of a host gene whose protein product is presumed essential for normal larval growth and development. The consequent block in the translation of an essential protein would be expected almost immediately to halt normal insect physiology. Antisense inhibition of a host gene has been previously observed in larvae infected with recombinant baculoviruses containing the juvenile hormone esterase gene under the polyhedrin or p10 promoters (Roelvink *et al*., 1992).

To test the efficacy of the antisense approach in increasing the insecticidal potential of baculoviruses, we selected a gene whose protein product is known to have a plurality of effects during development of vertebrates. The c-*myc* gene encodes a nuclear phosphoprotein which has been shown to be an important regulator in the determination of the various fates of a cell, namely, proliferation, arrest, differentiation and death (for an extensive review see Marcu *et al*., 1992 ), all of which are important regulatory events during development. The c-*myc* protein belongs to a growing family of transcription factors with a DNA-binding and dimerization domain of the helix–loop–helix–leucine zipper category (HLH-LZ motif) at the C terminus of the protein. The N terminus contains three highly conserved transcription activation domains, designated boxes A, B and C (for a short review see Krause & Luo, 1994).

While the gene has not yet been characterized in insects, its discovery in two invertebrate organisms, the sea star *Asterias vulgaris* (Walker *et al*., 1992) and the eastern oyster *Crassostrea virginica* (Marsch & Chen, 1995) suggests that its important functions extend to invertebrate evolution. The sea star equivalent of the c-Myc protein shows an overall amino acid conservation of 46% as compared to human c-Myc, but a much higher conservation (63–95%) within the transcription activating boxes A, B and C and the DNA-binding and oligomerization HLH-LZ domain (Walker *et al*., 1992). Because the highest conservation was found in the A, B and C domains, which correspond to the N terminus of the protein where an antisense RNA is more likely to impede translation initiation, we selected an exon II fragment of the human c-*myc* gene which contains these domains.

We constructed the recombinant viruses from the wild-type *Autographa californica* nucleopolyhedrovirus (AcMNPV) and report here the results obtained with infected *Spodoptera frugiperda* larvae. We also used human anti-c-Myc antibodies to explore the existence of a Myc-like protein in uninfected *S. frugiperda* cells and compared the relative levels of the protein as a function of time after infection with recombinant and control viruses.

## Methods

■ **Tissue culture cells, virus and vector construction.** Wild-type AcMNPV virus strain E2 and *S. frugiperda* Sf9 cells were obtained from M. Summers (Texas A&M University, USA). The cells were cultivated in Grace's insect medium (Gibco BRL) supplemented with 8% foetal bovine serum (FBS). Recombinant viruses were obtained by transfecting cells with wild-type virus DNA together with constructed plasmid vectors. Three plasmid recombinants were constructed from pBlueBacHis baculovirus vector (Invitrogen) as follows:

**(i) Human c-*myc* sense (Myc S).** A 754 bp sequence from c-*myc* exon II, bases 4650–5403 (Gazin *et al*., 1984), was cut with *Eco*RI and *Bam*HI and ligated to the *Bam*HI site of the pBlueBacHis vector, downstream of the polyhedrin promoter. The *Eco*RI end was filled with T4 DNA polymerase and the plasmid circularized by blunt-end ligation (Sambrook *et al*., 1989).

**(ii) Human c-*myc* antisense (Myc AS).** The *Bam*HI-cut end of the same c-*myc* fragment as above was ligated to the *Bgl*II end of the pBlueBacHis plasmid. The single-stranded ends were blunted and the plasmid recircularized as above. Sense and antisense recombinants could be distinguished because the *Bam*HI site is inactivated in the antisense but not in the sense construct while the *Hin*dIII site is inactivated only in the sense one (Fig. 1).

**(iii) Control c-*myc*-negative (Myc$^-$).** The pBlueBacHis plasmid was cut with *Bam*HI and *Hin*dIII to release the multiple cloning site, the digested DNA was then blunted and the plasmid recircularized as above.

All recombinant viruses were analysed by Southern blot hybridization to the appropriate probe to ensure that the correct fragment was present in the desired orientation. The probe used for c-Myc S and AS recombinants was a 201 bp fragment, residues 4799 to 4999 (Gazin *et al*., 1984), obtained by PCR using primers flanking box B of human exon II. The c-Myc$^-$ recombinant was probed with the $\beta$-galactosidase sequence of the pBlueBacHis vector, subcloned in our laboratory. Restriction enzyme digests and agarose gel electrophoresis were performed using standard procedures.

■ **Production and purification of recombinants.** Wild-type AcMNPV and constructed vector DNAs were cotransfected into Sf9 cells using Lipofectin (Gibco BRL) in serum-free medium, essentially as described by O'Reilly *et al*. (1992). Cell monolayers were washed twice with serum-free Grace's medium and the premixed Lipofectin–DNA mixture (1 μg virus DNA, 4 μg vector DNA, 30 μl Lipofectin, 1 ml serum-free medium) was added to each well of a 6-well plate with $7 \times 10^5$ cells/well. Cells were incubated at 25 °C for 4–8 h, the media was discarded and replaced with 2 ml of media with 10% FBS and incubated for 3 to 4 days. Recombinant viruses were selected by plaque assay as blue plaques after staining with X-Gal (Xymotech). Plaque purification was carried out at least three times to ensure virus homogeneity. All viruses were titrated by either plaque assay or endpoint dilution (O'Reilly *et al*., 1992).

■ **Northern blot analysis.** For Northern blot hybridizations, RNA was extracted from haemolymph, gut and fat body/cuticle pooled from 10 larvae at different times post-infection during bioassays (see below) using the guanidinium isothiocyanate method (Sambrook *et al*., 1989). The RNAs were separated in 1·2% formaldehyde–agarose gels, blotted to Biotrans membranes (ICN) and hybridized either to the *myc* probe described above for Southern hybridizations or to a probe for polyhedrin. Probes were labelled with $^{32}$P by standard procedures.

■ **Protein and immunoblot analysis.** Sf9 cells were cultivated as above and infected with recombinant viruses at an m.o.i. of 1 to 5 p.f.u./cell for 1 h at room temperature. The medium was replaced with fresh Grace's medium and cells were incubated for various time periods. For total cell extracts, cells were washed twice with ice-cold PBS, lysed directly in RIPA buffer (50 mM Tris–HCl pH 7·5, 150 mM NaCl, 1% NP-40, 0·5% sodium deoxycholate, 0·1% SDS) to which PMSF was added to prevent protein degradation. For nuclear and cytoplasmic fractions, cells were separated in cell lysis buffer (10 mM Tris–HCl

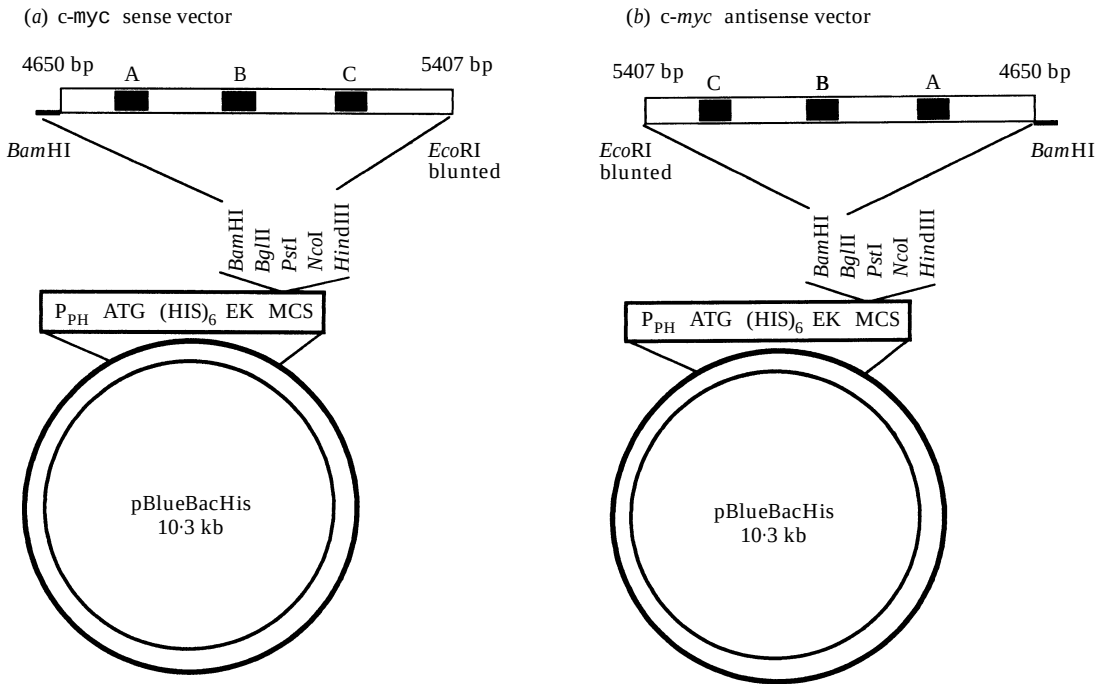


Fig. 1. Diagrammatic representation of the two c-*myc* recombinant vectors as derived from the pBlueBacHis plasmid (Invitrogen). C-*myc* transcription activation domains A, B and C are represented as black boxes.

pH 7·5, 150 mM NaCl, 5 mM $MgCl_2$, 0·5 % NP-40, 1 mM PMSF). The lysate was centrifuged for 5–10 min and the supernatant was taken as the cytoplasmic fraction while the nuclear pellet was lysed in RIPA/PMSF buffer as for the total extract above. After estimation of protein content by the Bradford method at 595 nm, an equal volume of 2 × SDS–PAGE sample buffer was added and the proteins were separated by electrophoresis in 8 to 12·5 % SDS–polyacrylamide gels (Laemmli, 1970). Gels were stained with Coomassie brilliant blue or blotted to PVDF Immobilon-P (Millipore) or nitrocellulose membranes. The blots were treated overnight at 4 °C with 5 % or 0·5 % (w/v) skimmed milk in TTBS (20 mM Tris–HCl pH 7·5, 200 mM NaCl, 0·1 % Tween 20) followed by an additional 1 h incubation at room temperature to block non-specific antibody binding. The membranes were then incubated with polyclonal human c-Myc antibody (Upstate Biotechnology) for 1·5 h at room temperature. Each membrane was washed three times, for 15 min each, with washing buffer (Kilkgaard & Perry Laboratories) or TTBS and incubated with 1:10 000 dilution of goat anti-rabbit IgG–horseradish peroxidase conjugate (Kilkgaard & Perry Laboratories) as a secondary antibody for 1·5 h at room temperature. Blots were washed again three times in TTBS, treated with chemiluminescent reagents (Kilkgaard & Perry Laboratories) for the detection of antibody binding and autoradiographed on X-ray film according to the manufacturer's instructions.

■ **Bioassays in *S. frugiperda* larvae.** For bioassays, a total of approximately 300 third and fourth instar larvae (average weight 115 mg) were injected with 2 µl each of either wild-type or recombinant viruses, all titred by endpoint dilution at $2 \times 10^8$ p.f.u./ml. A mock-infected control group was injected with 2 µl of plain Grace's medium. Larvae were kept in individual 1 oz plastic creamer cups provided daily with 300 mg/cup of fresh pinto bean diet pellets and scored for food consumption and survival as a function of days after treatment. Data were subjected to analysis of variance for significance of effects between virus treatments and covariant analysis for the contribution of individual larval weights.

## Results

### Rationale for recombinant virus construction

The exon II fragment of the human c-*myc* gene, containing the three conserved transcription activation domains of the protein, was inserted into the pBlueBacHis vector as detailed in Methods and illustrated in Fig. 1. Since this fragment was inserted, in both sense and antisense orientation, downstream of the promoter for the virus gene encoding the polyhedrin protein, all recombinants produced occlusion body-negative ($OB^-$) progeny. The polyhedrin late promoter was chosen for the following reasons: (i) it is activated only after virus DNA replication and therefore antisense inserts will not impede virus production; (ii) the stringent requirements for host factors in virus DNA replication restrict the host range of the virus, thus the inserts are unlikely to be expressed in any other untargeted organism; (iii) the polyhedrin promoter gives rise to abundant transcripts which are required to ensure success of the antisense strategy; (iv) the $OB^-$ strains of the virus are unstable and thus the recombinant virus will not persist in the environment. While virus fragility may be undesirable at later stages of application, it can be considered an additional safety feature for initial field testing.

### Analysis of virus RNAs produced in infected larvae

To interpret bioassay results, it is important to estimate the time of abundant production of polyhedrin promoter-driven transcripts in infected larval tissues for each experiment. To

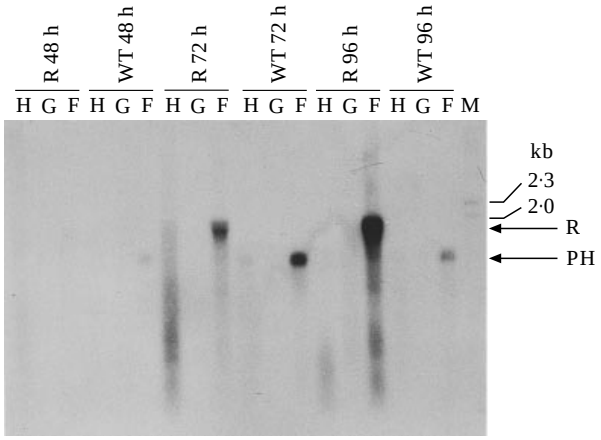


Fig. 2. Northern blot analysis of larval tissues obtained from wild-type-infected (WT) and human c-*myc* antisense recombinant-infected (R) *Spodoptera* larvae probed with the 201 bp fragment of the human c-*myc* gene and the AcMNPV polyhedrin sequence. H, haemolymph; G, gut; F, fat body; M, phage lambda *Hin*dIII markers. The positions of antisense recombinant (R) and polyhedrin transcripts (PH) are indicated by arrows and the times post-infection are given.

achieve this, RNA was purified from different tissues of 10 infected larvae at different days after infection and assayed by Northern blot hybridization as described in Methods. The results of one of the blots are illustrated in Fig. 2; only larvae infected with one of the recombinants (c-*myc* antisense virus) are shown as all recombinants yielded essentially similar results. We were unsuccessful in obtaining enough undegraded polyhedrin-driven transcripts from haemolymph or gut tissues. However, fat body and associated tissues consistently showed abundant transcripts by 72 h post-infection. Therefore, we would expect an effect, if any, of the antisense c-*myc* insert by this time. Compared to wild-type, the recombinant-infected fat bodies showed a larger transcript due to insertion of the 754 bp human c-*myc* fragment, which disrupts the polyhedrin gene ORF. Quantitative differences shown here between wild-type and recombinant viruses at 96 h post-infection were not consistently observed and were probably due to differences in loading.

## Protein analysis and Western blotting with human c-Myc antibody

To confirm the expected block in translation of the polyhedrin protein, Sf9 cells infected with wild-type and all recombinant viruses were analysed by SDS–PAGE. At a high m.o.i. in the cell cultures, polyhedrin protein could be detected in wild-type infections as early as 36–48 h post-infection but appeared highest at 72 h (data not shown). As expected, no polyhedrin protein could be seen in any recombinant-infected cells, which instead produced the *β*-galactosidase protein from the virus ETL promoter in the vector.

To determine whether any conservation exists between our human c-*myc* sequences and endogenous *S. frugiperda* transcripts, we first attempted Northern blot hybridizations with human c-*myc* exon II probes. However, we could not detect reliable levels of transcripts. This is not surprising, given that c-*myc* mRNAs of untransformed cells are also very difficult to detect in Northern blots as they are relatively rare (Gallant *et al.*, 1989). C-*myc* mRNA detection can be best achieved after RT–PCR (Luo & Krause, 1994). Since RT–PCR is unreliable in the absence of appropriate insect sequence information, we searched for a c-Myc-like endogenous protein through the use of a polyclonal human c-Myc antibody, which might detect conserved conformational structures between human and insect proteins.

As can be seen in Fig. 3 (*a*), a single strong band of approximately 67 kDa was apparent in the nuclear fraction of Sf9 cells. This is larger than the 60–62 kDa major protein seen

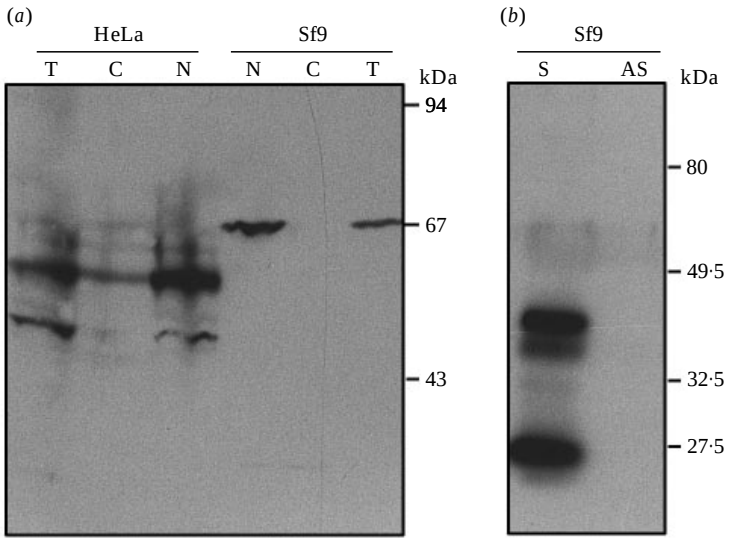


Fig. 3. Western blot analysis of proteins binding to human polyclonal c-Myc antibody. (*a*) Total (T), nuclear (N) and cytoplasmic (C) proteins of HeLa and Sf9 cells were loaded on 8% SDS–polyacrylamide gels and blotted as described in Methods. (*b*) Sf9 cells were infected with either sense (S) or antisense (AS) c-*myc* recombinant viruses and collected 72 h after infection. The positions of protein size markers are indicated.

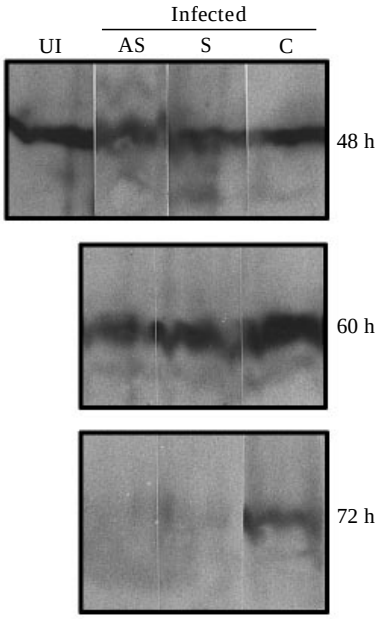


Fig. 4. Western blot of human c-Myc antibody binding to an Sf9 cell protein at different times after infection with recombinant viruses. UI, uninfected cells; AS, infected with c-*myc* antisense recombinant; S, infected with c-*myc* sense recombinant; C, infected with control Myc$^-$ recombinant. The times post-infection with 1 p.f.u./cell of virus are shown on the right. For each infection time, the same amount of protein was loaded in each well.

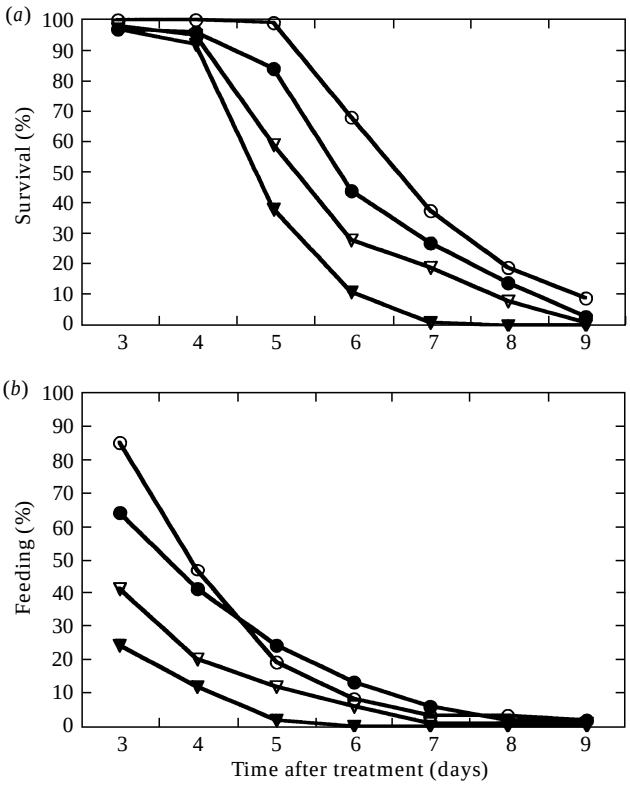


Fig. 5. Results of bioassays performed in *Spodoptera* larvae injected with 2 µl ($2 \times 10^8$ p.f.u./ml) each of wild-type (●), human c-*myc* antisense (▼), human c-*myc* sense (▽) and control (○) recombinant viruses. (*a*) Percentage survival times following treatment. (*b*) Percentage feeding times. In both (*a*) and (*b*) the curves show scores for 300 larvae, average weight 115 mg for each treatment, as explained in Methods. Mock-infected larvae developed and fed normally and are therefore not included in the graphs. For the same reason, days 1 and 2 are not included as normal scores were registered on both days.

in the human HeLa cell lanes. Fig. 3 (*b*) shows the absence of the 67 kDa band in Sf9 cells after 72 h infection with recombinant c-*myc* sense and antisense viruses, indicating virus interference with cellular gene expression. However, the sense recombinant viruses showed two major bands (Fig. 3*b*), the smaller corresponding to the human exon II fragment inserted into the polyhedrin sequence (26 kDa translated from the c-*myc* start codon) and the larger, of about 38 kDa, to the translation product starting from the upstream ORF within the vector. Although we did not intend to insert the c-*myc* sense sequences in frame with the vector translation start site, the appearance of these peptides helped us to estimate the time of activation of the polyhedrin promoter in OB$^-$ recombinants. However, expression of truncated c-Myc peptides in the sense recombinant might interfere with host metabolic processes and therefore a third control recombinant virus, containing no insert, was deemed essential for interpretation of results.

We next investigated whether any differences in the relative amounts of this c-Myc-like endogenous protein could be detected in cells infected with either sense or antisense c-*myc* recombinant viruses, as opposed to c-*myc*-negative control virus. We used each recombinant virus at a maximum m.o.i. of 1 p.f.u./cell to slow down the infection process for improved detectability of any differences between treatments. Fig. 4 represents one of many blots, all of which showed the same relative differences between recombinant and control viruses. Although little or no difference could be detected between c-*myc* sense and antisense recombinants, the c-Myc-like cellular band disappeared consistently faster in cells infected with these viruses than in those infected with the control. Our interpretation of these results will be discussed below.

### Bioassays in *S. frugiperda* larvae

The final test was to determine the effects of wild-type and recombinant viruses on food consumption and survival of infected larvae. Third to fourth instar larvae were injected with each virus at an average dose of 3500 p.f.u./mg body weight as described in Methods. Population dynamics involving a large combined bioassay of 300 larvae (average size 115 mg) for each virus treatment are illustrated in Fig. 5. Fig. 5 (*a*) illustrates the time to death as a percentage of surviving larvae while Fig. 5 (*b*) shows the effects on food consumption as a percentage of the larvae showing normal feeding. The data were plotted starting on day 3, since this is the time polyhedrin promoter-driven transcripts were seen to accumulate (Fig. 2) and all larvae fed normally for the first 2 days after treatment. Larvae interrupt feeding as they start to moult but resume

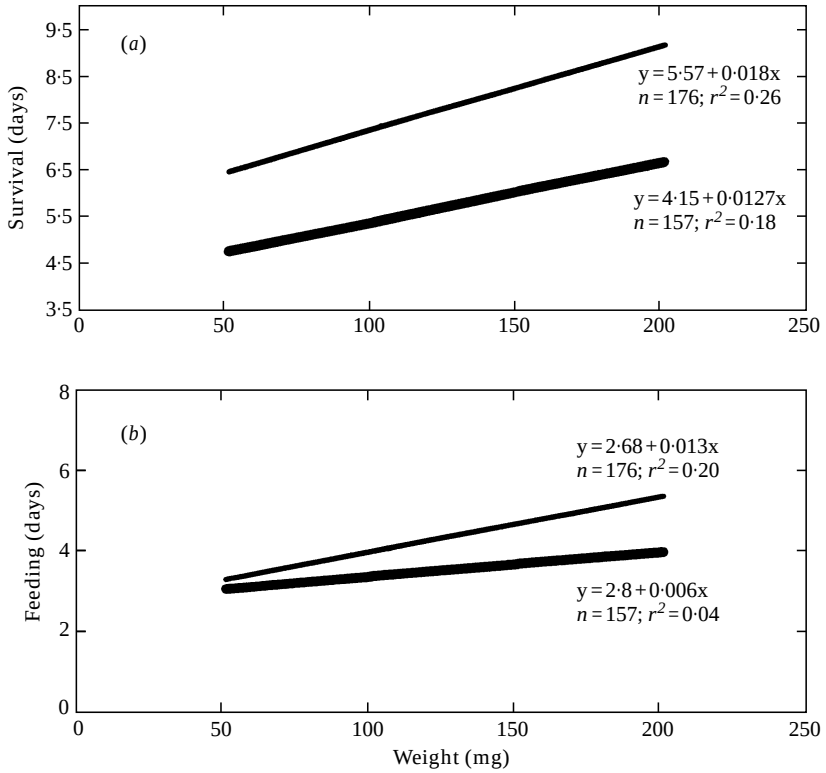


Fig. 6. Survival and feeding times as a function of weight of larvae infected with Myc AS recombinant (thick line) and control Myc⁻ (thin line) viruses. Number of larvae analysed for each treatment ($n$) and coefficients of determination ($r^2$) are indicated.

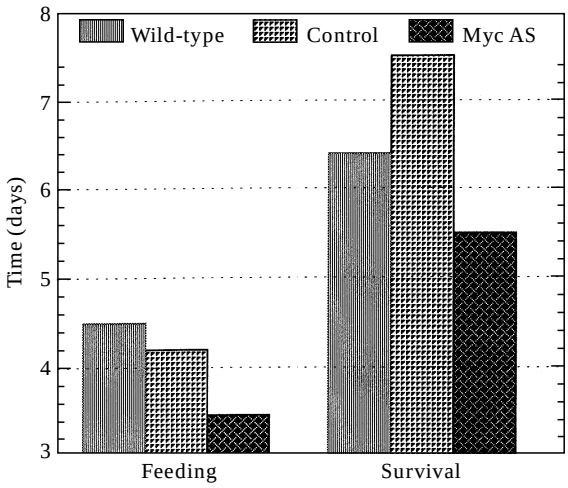


Fig. 7. Average treatment and survival time of larvae infected with antisense c-*myc* (Myc AS), Myc⁻ (control) and wild-type viruses. Differences among treatments were significant at $P < 0{\cdot}001$.

feeding normally immediately after and were, therefore, scored as normal. However, many of the wild-type and recombinant virus-infected larvae did not resume feeding, becoming stuck in the moulting process and were thus scored as abnormal. All mock-infected larvae fed normally and survived to pupate and are, therefore, not represented in either graph.

The survival dynamics presented in Fig. 5 (*a*) show increasing relative effects of control, wild-type, sense and antisense c-*myc* recombinant viruses, in that order. Time to death in larvae infected with the sense recombinant virus appears intermediate between that of antisense and wild-type viruses, indicating some interference of the c-*myc* sense transcripts with host developmental processes. It is evident from Fig. 5 (*b*) that 75% of the larvae infected with antisense c-*myc* virus stop feeding almost as soon as the polyhedrin promoter-driven transcripts began to accumulate in the larvae (Fig. 2). Only 15% of the larvae infected with the control c-*myc*-negative virus stopped feeding at this time. Again, larvae infected with the c-*myc* sense recombinant appeared to be affected to some extent compared to those infected with wild-type and control viruses. These results, together with those obtained in the Western blot analysis above, indicate that the c-*myc* sense recombinant virus is inappropriate as a control for the effect of the antisense transcripts, thus the OB⁻ myc-negative recombinant virus represents the best control for the effects of virus recombinants bearing any insert.

Although we obtained consistent relative results comparing the four virus treatments in three separate bioassays, we noticed that the smaller the larvae, the stronger the effect. To test this statistically, we used groups of larvae with pre-treatment weights evenly distributed between 40 and 200 mg. Both survival and feeding time increased with the weight of the larvae. This relationship is quantified for two of the treatments (Myc⁻ control and Myc AS) in Fig. 6 (*a*, *b*). The difference in slope indicated that the effect of larval weight on survival or feeding response was lowered in the Myc AS-

infected population. Of further interest are the coefficients of determination ($r^2$). In the population infected with control virus, 26% of the variance in survival time and 20% in feeding time was explained by the variation in weight, while the values for the Myc AS-infected population were only 18% and 4%, respectively. Given the large number for $n$, the regressions are statistically highly significant ($P < 0{\cdot}005$).

Following up on this information, the data from the various populations were tested by covariant analysis using larval weight as the covariate and average time of feeding and survival time as response variables. Both covariate and treatments were highly significant ($P < 0{\cdot}001$). Mean feeding time and survival are shown for wild-type-, Myc$^-$ control- and Myc AS-treated larvae in Fig. 7. It is apparent that the largest gain provided by the Myc AS recombinant virus as compared with the wild-type one, comes from arrest in larval feeding, which should result in drastically reduced crop damage.

## Discussion

### Evidence for a c-Myc-like protein in insects

To understand fully the multiple activities of the c-Myc protein, it is important to analyse and compare its structure and sequence conservation in as wide a variety of organisms as possible. Prior to its discovery in the sea star (Walker *et al.*, 1992), it was thought that c-Myc was restricted to vertebrates. Once that notion was dispelled, several groups attempted to characterize *myc* genes in other invertebrates (see Meichle *et al.*, 1992; Marsch & Chen, 1995). However, *myc*-like genes have yet to be characterized in insects despite early reports in *Drosophila* (Madhavan *et al.*, 1985). We thought that the more traditional approaches to finding a gene in an unrelated organism may not be sensitive enough in this case. We therefore looked for a more sensitive, even if indirect, indication of its existence. The baculovirus ability to express large quantities of any sequence inserted under either of its two late promoters, P10 or polyhedrin, could supply the necessary sensitivity. If an essential gene, with conserved sequence elements of the mammalian *myc* gene, does exist in insects, the abundant viral antisense transcripts would be likely to block translation of the endogenous mRNA resulting in an abnormal larval response.

Western blot analysis provided the first clue. The consistent finding of a single protein band in the nuclear fraction of Sf9 cells, which bound specifically to human c-Myc antibody, suggested the existence of a c-Myc equivalent protein in these cells. To test whether the antisense strategy works, we needed to find out whether this protein was specifically targeted by the c-*myc* recombinant viruses. This turned out to be the case as illustrated in Western blots of cells infected with c-*myc* recombinant and control viruses at various times post-infection. We did not initially expect that the c-*myc* sense recombinant would have any effect. However, it is likely that the truncated and over-expressed sense transcripts have a competitive effect in the normal processing and/or translation of the corresponding host mRNAs. This phenomenon, previously termed sense suppression or post-transcriptional silencing by exogenous mRNAs, has been detected in other systems such as plants (Flavell, 1994; Meins & Kunz, 1994; Carvalho-Niebel *et al.*, 1995; Ingelbrecht *et al.*, 1994; Matzke & Matzke, 1995; Jorgensen, 1995). Further indication of a similar effect in animals was reported more recently as a dominant negative mutation in myotonic dystrophy (Research News, *Science*, Nov. 17, 1995, p. 1120). Thus, while the effects of the c-*myc* sense recombinant virus are not as dramatic as those observed with the antisense recombinant on either larval feeding or survival times, one would expect that its interference with the synthesis of a corresponding host protein will result in some degree of inhibition of normal larval development. Alternatively, although less likely, the truncated human c-Myc peptide translated from the sense insert may have a competitive effect in relation to the normal role of the corresponding endogenous protein.

It could be argued that the late synthesis of antisense transcripts may be unlikely to have much of an effect on cellular proteins, most of which are known to be severely depressed by the time the virus late promoters become activated. This may be the case for most proteins which are no longer needed by the virus. However, our immunoblots detect the c-Myc-like nuclear protein as being one of those that persist in cells infected with the control virus. Presumably this protein, like the c-Myc protein of vertebrates, may be active not only in DNA replication, but in modulating apoptosis (Harrington *et al.*, 1994), effects which may favour virus reproduction and release, respectively. Such proteins would likely be selected for longer preservation during virus infection.

### The antisense strategy applied to insect pest control

We have made use of a simple antisense strategy in an attempt to increase baculovirus effectiveness in insect pest control. The choice of the c-*myc* gene as a target for inhibition of its expression by antisense RNA was, in part, determined by our interest in exploring the likelihood of its existence in insects. However, any gene essential for larval growth and development might be as effective a target for such a strategy, provided the particular gene product normally persists beyond the time of activation of the promoter chosen to drive expression of the antisense insert. Thus, the path is open for trials designed to test the efficacy of any other gene in the process. Likewise, our choice of the polyhedrin promoter to drive transcription of antisense RNA may be questioned on the basis that spray programmes in the field would have little chance of success when using the fragile OB$^-$ viruses. However, safety considerations dictate that, at least for initial trials, one must ensure that the recombinant viruses will not persist in the environment. For pesticide development one might either place the antisense fragment downstream of

another strong virus late promoter, such as P10, or coproduce recombinant and wild-type virus such that the polyhedrin protein will be provided *in trans* to the recombinants in double-infected hosts. In such mixed populations only the wild-type viruses are likely to survive in future years (Groner, 1986). The latter approach may be preferable since inactivation of the *p10* gene is also known to result in pitted and fragile polyhedra (Gross *et al*., 1994).

The present results indicate that the antisense recombinant virus is highly efficient in stopping larval feeding almost immediately following activation of the polyhedrin promoter which produces the antisense transcripts. Death of the infected larvae follows approximately 2 days later. This is in contrast to the effect of wild-type viruses. While wild-type viruses are also effective killers, they are slow and infected larvae continue feeding beyond the time when uninfected larvae stop to pupate.

Our clear demonstration of the increased susceptibility of small larvae to virus infection supports early biopesticide application, when the larval populations have just started to feed. The 3 day delay in response imposed by the choice of a late promoter can, therefore, be offset by the stronger inhibitory effect. This means that early applications of recombinant viruses to emerging larval populations should be quite effective in inhibiting crop damage. The alternative of introducing the antisense inserts under an early virus or cellular constitutive promoter might appear more promising but would be self-defeating. Not only would those promoters be unlikely to produce the required large excess of antisense transcripts, but premature cell death would threaten virus replication, affecting virus biopesticide production. Furthermore, virus host range selectivity would probably be lost, resulting in possible infection of beneficial insects.

Many reports have shown success in improving the insecticidal effectiveness of baculoviruses, either through the expression of insecticidal proteins, neurotoxins or juvenile hormone inhibitors (Carbonell *et al*., 1988; Merryweather *et al*., 1990; Tomalski & Miller, 1991; Stewart *et al*., 1991; Maeda *et al*., 1991; Tomalski *et al*., 1993; Ribeiro & Crook, 1993; Cory *et al*., 1994; Chejanovsky *et al*., 1995; Eldridge *et al*., 1995; Heinz *et al*., 1995). However, concerns remain over possible effects on non-target organisms as well as acquired insect resistance to introduced foreign proteins.

As compared with the previous reports, our strategy has two important advantages: one is that the virus recombinant is simple to construct since it does not require a complete gene whose expression, processing or secretion must be regulated. Several reports have pointed out difficulties in ensuring correct secretion (Vakharia *et al*., 1995) or processing of peptides introduced into baculovirus genomes (Bozon *et al*., 1995). The other advantage is that, in the absence of any ORF within the inserted sequences, no foreign protein is being produced in the infected larvae and therefore insect resistance is unlikely to develop. The potential for the development of resistance against introduced foreign proteins has been known since 1985 and demonstrated in the case of *B. thuringiensis*, where it is increasingly recognized as a problem in the implementation of *B. thuringiensis* spray programmes for the control of agricultural and forest pest populations (McGaughey, 1994). In contrast, the baculovirus antisense approach (Canadian patent file 2 177 509) should not only avoid this problem but, by targeting only particular pests, also avoid damage to beneficial insects.

While further research is necessary to characterize the cellular target of the antisense transcripts and to establish the precise mechanism of its insecticidal activity, this report strongly suggests that the antisense strategy is a powerful tool for the engineering of baculovirus-based insecticides.

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