Male sterility in *Heliothis virescens* × *H. subflexa* backcross hybrids

Evidence for abnormal mitochondrial transcripts in testes

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Summary. Interspecific matings between *Heliothis virescens* males and *H. subflexa* females result in the production of progeny normal in all respects except that the males are sterile. Recurrent backcrossing of fertile females to *H. virescens* males perpetuates testis dysfunction in these lines. Although the basis of this phenomenon is unknown, characteristic degenerative abnormalities in sperm mitochondria from backcross males suggest that the normal function of this organelle is disrupted. In order to test this notion, mitochondrial gene sequences have been isolated from a cDNA library constructed from *H. virescens* testis poly (A) + RNA, aligned on the mitochondrial DNA physical map, and assigned genetic designations based upon their homology with sequenced mouse mitochondrial DNA restriction fragments. Hybridizations of these DNA sequences to RNA molecules from *H. virescens* and backcross testes revealed two major differences between the two lines: (1) the steady-state levels of mitochondrial transcripts from backcross testes were reduced approximately 3-fold relative to *H. virescens*; and (2) transcripts encoding mitochondrial proteins were not polyadenylated in backcross testes. Neither of these abnormalities appeared in mitochondrial transcripts from other tissues in backcross moths. These findings suggest that abnormal mitochondrial RNA metabolism is either a direct cause, or a manifestation of, the mechanism of sterility in backcross males.

Key words: *Heliothis* interspecific matings – Male sterility – Sperm mitochondria – Mitochondrial transcripts – RNA metabolism

Introduction

Interspecific matings between *Heliothis virescens* males and *H. subflexa* females result in the production of viable progeny. However, while the *F₁* females prove to be fully fertile when backcrossed to both parental males, *F₁* males produce no offspring when mated to females of either species (Laster 1972). Repeated matings of fertile hybrid females to *H. virescens* males give rise to successive backcross generations, all the males of which continue to exhibit complete sterility. Indeed, backcross lines maintained for over 100 generations continue to produce sterile male moths (Laster et al. 1985), and their release into the field promises to be of great practical importance in suppressing endemic populations of *H. virescens* (Mackla and Huettel 1979; Proshold 1983).

While there is no information available concerning the mechanistic basis of persistent male sterility in these backcross progeny, it is nonetheless apparent that their reproductive phenotype is not determined solely by chromosomal complement. First, sperm from later backcross generation males continue to be inviable in spite of the segregation of *H. subflexa* chromosomes and their replacement with *H. virescens* homologues (LaChance 1984). And second, lines established via the reciprocal cross (*H. subflexa* males × *H. virescens* females) and maintained by backcrossing hybrid females to *H. subflexa* males, produce sterile males for only about five generations (Goodpasture et al. 1980a). Although sterility in these early backcross generation males may be attributable to desynapsis of nonhomologous chromosomes (Goodpasture et al. 1980a, b), fertility is restored in a presumably homogeneous chromosomal background. These observations suggest that cytoplasmically inherited factors play a central role in modulating the development of sperm cells.

Two general classes of cytoplasmically transmitted determinants have been linked to male infertility in other systems. First, sexually transmitted microbial agents (streptococcal t-forms) have been demonstrated to cause male sterility in *F₁* hybrids between subspecies of *Drosophila paulistorum* (Somerson et al. 1984). This explanation is untenable in the case of *Heliothis* hybrid male sterility since male fertility is not restored in backcross lines by either antibiotic, antiviral or temperature treatments (LaChance and Karpenko 1981, 1983). Second, cytoplasmic male sterility in *Zea mays* may be attributable to the apparently abnormal function of the mitochondrial genome in certain nuclear backgrounds (Leaver 1980; Laughnan and Gabay-Laughnan 1983). Male sterility in this system manifests itself in a number of distinct biochemical phenotypes including physical alterations in mitochondrial translation products.
(Forde et al. 1978), and changes in the structure of mitochondrial DNA (Pring and Levings 1978; Kemble and Thompson 1982).

Circumstantial evidence points to a mitochondrial involvement in Heliothis hybrid male sterility as well. Backcross moths (both male and female) possess the mitochondrial genome of the original H. subflexa mother (Lansman et al. 1983) in a background of H. virescens chromosomes. Also, the morphological abnormalities seen in sperm from backcross testes primarily involve the mitochondrial derivatives (Goodpasture et al. 1980a; LaChance 1984; Miller and Huettel 1986). A working hypothesis which accounts for persistent male sterility in backcross hybrids has been formulated (Laster et al. 1985), namely, that a mitochondrial function peculiar to sperm cells is impaired in backcross moths due to the inability of evolutionarily divergent nuclearly encoded (H. virescens) and mitochondrially encoded (H. subflexa) RNA or protein molecules to interact appropriately.

As a first step in evaluating the function of sperm mitochondria in fertile and sterile males we have chosen to examine the structure of mitochondrial genes and their transcripts. Here we report on the isolation of cDNA sequences corresponding to specific mitochondrial genes and on analyses of the structures and levels of their corresponding transcripts in testes. At least three mitochondrial RNAs were found not to be polyadenylated in backcross testes, and also reduced in amount relative to H. virescens. The significance of these biochemical abnormalities is discussed in terms of the possible mechanistic basis of hybrid male sterility.

**Materials and methods**

**Insects.** The H. virescens and backcross lines used in these experiments were reared according to standard procedures (Proshold and Bartell 1970). Both lines were originally established at the Bioenvironmental Insect Control Laboratory, Stoneville, MS, USA, and were transferred to our laboratory in 1979. The backcross males used in these studies represented the 50th to 55th backcross generations.

**DNA isolation and manipulations.** Total DNA was isolated from sperm as described by Maniatis et al. (1982) for the preparation of high molecular weight genomic DNA. Mitochondrial DNA was prepared according to the procedure of Lansman and Clayton (1975) by sedimentation in cesium chloride gradients containing ethidium bromide. The mitochondria used in these preparations were obtained from whole fourth or early fifth instar larvae via differential centrifugation as described elsewhere (Miller and Huettel 1986). DNA molecules were cleaved with restriction endonucleases according to the supplier's recommendations (Miller and Huettel 1986). DNA fragments were transferred to nitrocellulose according to the procedure of Lansman and Clayton (1975) by sedimentation in cesium chloride gradients containing ethidium bromide. The mitochondria used in these preparations were obtained from whole fourth or early fifth instar larvae via differential centrifugation as described elsewhere (Miller and Huettel 1986). DNA molecules were cleaved with restriction endonucleases according to the supplier's recommendations (Boehringer-Mannheim, FRG) and fractionated using agarose gels prepared with Tris-acetate/EDTA buffer (Maniatis et al. 1982). DNA fragments were transferred to nitrocellulose according to Southern (1975). DNA filters were prehybridized for 6–12 h at 65°C in 6 x SSC, 0.5% SDS, 5 x Denhardt’s solution, and 100 µg/ml sheared salmon sperm DNA, and then incubated with radiolabeled probe for 20–24 h at 65°C in the same solution. The filters were washed in 2 x SSC/0.1% SDS (twice for 15 min at 25°C, then three times for 30 min at 65°C). Hybridization probes were prepared by nick translation of DNA molecules as described by Rigby et al. (1977) using [32P]-dCTP (New England Nuclear, Boston, MA, USA). Where indicated, gel-fractionated DNA molecules were eluted and purified from agarose fragments according to Benton (1984).

**RNA isolation and manipulation.** RNA molecules were isolated from various dissected tissues by homogenization in guanidine isothiocyanate (Chirgwin et al. 1979) and centrifugation through CsCl (Maniatis et al. 1982). Polyadenylated RNA was isolated via chromatography on oligo (dT)-cellulose (BRL) using the salt conditions described by Maniatis et al. (1982).

Unfractionated RNA preparations were dot-blotted onto nitrocellulose filters in the presence of saturated sodium iodide at 25°C as described by Bresser et al. (1983) and Gillespie and Bresser (1983). Subsequent washing and fixing of filter-bound RNA was performed exactly as described by these authors. The filters were both prehybridized (20-24 h) and hybridized (40-48 h) at 42°C in 50% formamide, 5 x SSC, 5 x Denhardt’s solution, 50 µg/ml sheared salmon sperm DNA, 50 µg/ml polyadenosine and 1% SDS. The filters were washed three times at 42°C (30 min each) in 10 x SSC/0.1% SDS and then one time (60 min) at 60°C in 0.1 x SSC/1% SDS.

Poly (A)− and poly (A)+ RNA samples were treated with glyoxal, separated on 1.5% agarose gels, and blotted onto nitrocellulose as described by Thomas (1980, 1983). Filters were both prehybridized (20 h) and hybridized (24 h) at 42°C in 50% formamide, 5 x SSC, 5 x Denhardt’s solution, 200 µg/ml sheared salmon sperm DNA, 20 µg/ml polyadenosine and 0.2% SDS. Washes consisted of 2 x SSC/0.1% SDS (two times for 15 min at 25°C, then twice for 15 min at 50°C) and 0.1 x SSC/0.1% SDS (twice for 30 min at 50°C).

**cDNA library construction and colony hybridizations.** Poly (A)− RNA isolated from H. virescens late 5th instar testes was used to construct a cDNA library. The procedures used for synthesizing and cloning double stranded deoxyctydine-tailed cDNA molecules are described in detail by Maniatis et al. (1982). Colony hybridizations and screening with [32P]-dCTP-labeled mitochondrial DNA were performed according to Hanahan and Meselson (1980).

**Isolation of plasmid DNA and inserts.** Plasmid DNAs were isolated using the alkaline lysis protocol described in Maniatis et al. (1982). Cloned cDNA contained in recombinant plasmids was isolated by cleavage with PstI (BRL, Gaithersburg, MD, USA) followed by electrophoresis on 4.5% polyacrylamide gels (Maniatis et al. 1982). Fragments of interest were localized by staining with ethidium bromide, excised from the gel, and the DNA eluted with 0.5 M Na-acetate, pH 6, 1 mM EDTA, 10 µg/ml E. coli tRNA for 16 h at 37°C.

**Measurements of protein synthesis.** Testes were dissected from staged H. virescens and backcross pupae, rinsed in sterile Grace’s medium (Gibco, New York, NY, USA), and the cells released into medium by rupturing the testis sheath with forceps. The cells from three individuals were pooled, pelleted by centrifugation for 10 s at 1000 x g, and resuspended in 250 µl of Grace’s medium lacking t-methionine. Samples used for determinations of total cellular protein synthesis received 10 µCi [35S]-methionine (1063 Ci/mmole;