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Evidence for a Chromosomal Location of Polydnavirus DNA in the Ichneumonid Parasitoid *Hyposoter fugitivus*

DEMING XU AND DON STOLTZ*

Department of Microbiology, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

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Evidence is presented in support of a chromosomal location for sequences homologous to polydnavirus DNA in the ichneumonid parasitoid *Hyposoter fugitivus*. In this study, four different viral genome segments were cloned and used as probes against genomic DNA extracted from male parasitoids and digested with a variety of restriction enzymes. Each probe typically identified a single off-size fragment (OSF) in the case of enzymes not cutting viral genome segments, while two OSFs were generated by enzymes cutting at one and two sites. While extra OSFs were occasionally observed, these were invariably found to be due to the presence of polymorphic restriction sites in flanking chromosomal DNA. Analysis of these data suggests that a single, stable chromosomal locus exists for sequences homologous to each viral genome segment; the data also indicate that viral and cognate parasitoid genomic DNAs are largely if not entirely colinear.

The polydnaviruses comprise a large group of viruses defined principally on the basis of having segmented double-stranded circular DNA genomes (12). At present, two groups are recognized: the bracoviruses, which have cylindrical nucleocapsids surrounded by a single unit membrane envelope, and the ichnoviruses, in which fusiform nucleocapsids are surrounded by two unit membrane envelopes (the names are derived from the parasitoid families Braconidae and Ichneumonidae, in which these viruses respectively replicate). One particularly unusual feature of these viruses is their invariant presence in the ovaries of all females of all affected species (13); that is, transmission in parasitoid populations occurs with 100% efficiency. Considering that polydnaviruses are known to be required for successful parasitism (1, 10), the importance of an efficient mode of transmission becomes quite obvious. In an earlier study (11), we argued that per os transmission, the most common mode of transmission for insect viruses, would not likely occur with anything approaching that efficiency; furthermore, we presented experimental evidence against a per os transmission mode. At the same time, we showed that polydnavirus DNA could be transmitted vertically through germ line tissue. Contemporary work by Fleming and Summers (3) indicated a probable chromosomal location for two *Campoletis sonorensis* polydnavirus (CsPV) genome segments; these authors suggested that chromosomal integration could provide a physical basis for polydnavirus transmission but were unable to rule out the possibility that extrachromosomal viral DNA circles (known to be present in many, if not all, parasitoid tissues) might be involved in the transmission of viral genomes. More recently, a genetic approach to this question was developed, in which isofemale parasitoid lines carrying slightly different polydnavirus genomes were used to study the segregation of genome segments into F_1 and backcross progeny (8). The results of that study demonstrated an apparent Mendelian transmission mode in the case of two unrelated polydnaviruses; it was concluded that extrachromosomal genome segments, even if present in the ovary prior to the onset of viral DNA replication, probably do not contribute to the structure of encapsidated polydnavirus genomes. The genetic approach, in short, suggested not only that chromosomally located homologs of polydnavirus DNA should exist, but also that these would be solely responsible for the transmission of polydnavirus genomes. It follows that chromosomal sequences homologous to polydnavirus genome segments may be involved in the replication of polydnavirus DNA.

It now becomes important to determine the location and structure of these regions within the parasitoid genome. Fleming and Summers have reported that chromosomal DNA from the ichneumonid parasitoid *C. sonorensis* contains sequences homologous to, and colinear with, two different CsPV genome segments (3, 4); furthermore, these authors have suggested that CsPV DNAs are stably integrated within the parasitoid genome, without significant rearrangement. Here, we present similar data in support of a chromosomal location for the genome of an unrelated ichnovirus, HfPV, from *Hyposoter fugitivus*. In this study, four different HfPV genome segments were used as probes versus genomic DNA extracted from male wasp parasitoids. Each probe typically identified only one or two off-size fragments (OSFs) in parasitoid DNA digested with a variety of restriction endonucleases. Our observations indicate that sequences homologous to encapsidated HfPV genome segments are stably located at single loci within the *H. fugitivus* genome.

MATERIALS AND METHODS

Insects and virus. The ichneumonid parasitoid (*H. fugitivus*) and its host, the forest tent caterpillar (*Malacosoma disstria*), were reared as described previously (11). Our present *H. fugitivus* colony is not considered to be inbred, having been supplemented during each of the past 3 years by field-collected material. Adult male wasps were periodically frozen by immersion in liquid nitrogen and subsequently stored at −70°C. Virus and viral DNA were purified as described previously (6, 11). It should be noted that viral DNA extracted from calyx fluid is only minimally contaminated with parasitoid chromosomal DNA; thus, in the blotting work described here, it was found that identical results could be obtained with use of either virus or calyx fluid.

* Corresponding author.

Parasitoid genomic DNA. Genomic DNA was extracted from pooled male wasps, using a guanidinium isothiocyanate protocol (7). Briefly, approximately 300 wasps were ground under liquid nitrogen to a fine powder, which was then suspended in 20 ml of 4 M guanidinium isothiocyanate containing 50 mM Tris-HCl (pH 7.6) 10 mM EDTA, and 2% sodium lauryl sarcosinate. Following removal of cellular debris by low-speed centrifugation (1,000 × g, 5 min), the supernatant was extracted successively with phenol and then extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1). To the aqueous phase, an equal volume of isopropanol containing and 1/20 volume 5 M NaCl was added in order to precipitate total nucleic acid. The nucleic acid precipitate was removed, washed in 50% isopropanol, and then dissolved in 10 ml of 10× TE (1× TE is 10 mM Tris-HCl plus 1 mM EDTA, pH 8). After successive digestion with protease K (100 μg/ml in 0.5% sodium dodecyl sulfate [SDS] at 37°C overnight) and RNase A (50 μg/ml at 37°C for 3 h), the nucleic acid remaining was phenol extracted and then precipitated with ethanol; the pellet was dissolved in 5 ml of 10× TE. The DNA solution was placed into a dialysis tube and concentrated to approximately 1 ml, using sucrose; subsequent dialysis was against 1× TE for 48 h. Using this procedure, we were routinely able to recover approximately 1 μg of DNA per male wasp.

Cloning strategy. Preliminary work strongly suggested that most if not all HfPV DNA bands seen following ethidium bromide staining contained more than one comigrating circular species. For this reason, we did not attempt to clone entire DNA circles by the method described by Theilmann and Summers (15). Instead, we developed a two-stage procedure in which inserts >2 kbp in size were selected at random from an initial *Hin*dIII library of viral DNA cloned in pUC19; selection was on the basis of strong hybridization to whole viral DNA. Inserts were then used as probes versus Southern blots of DNA digested by at least 12 different restriction enzymes. In some cases, the insert used was found to be equivalent in size to the genome segment to which it hybridized, suggesting that a complete DNA circle might have been cloned. More often, the insert identified a larger circle; however, an examination of the positions of hybridization signals in these cases almost invariably identified one or more enzymes cutting the circle in question once (referred to as one-cutters). A second library, using an identified one-cutter, was then constructed and screened with the previously cloned insert in order to isolate the desired genome segment. The advantage of this protocol is that it does not require that a particular genome segment be unambiguously identifiable within the entire viral genome following ethidium bromide staining. Almost 30 different viral DNA circles have now been cloned; in the case of the circles used in this study, cloning of complete genome segments was confirmed by comparison of restriction maps derived from circles cloned by using two or more different restriction enzymes.

DNA labelling and blotting. Standard DNA manipulations (electrophoresis, Southern blot hybridization, and restriction enzyme digestion) were essentially as described by Maniatis et al. (7) with minor modifications. Vector-free inserts to be used as probes were generated from recombinant plasmids by restriction enzyme digestion, followed by band extraction from agarose gels using GeneClean as recommended by the manufacturer (Bio 101, Inc., La Jolla, Calif.). Random primer labelling with [^{32}P]dATP was done by the method of Feinberg and Vogelstein (2). Nylon blots were prehybridized for 2.5 h at 68°C in 0.5 M sodium phosphate (pH 7.2) containing 7% SDS. Blots of wasp genomic DNA were hybridized with 1.5×10^6 cpm of probe per ml. For blots of viral DNA, 2.5×10^5 cpm of probe per ml was used. Following hybridization at 68°C overnight, blots were washed at 68°C in 50 mM sodium phosphate (pH 7.2) containing 1% SDS. Autoradiography using Kodak X-Omat film was at −80°C. Exposure times for the pictured blots are given in the figure legends. When required, blots were reused following removal of the previous probe by boiling for 30 min in 1× TE buffer containing 1% SDS.

Nomenclature of genome segments. The protocol currently followed in the literature assigns capital letters to each polydnavirus genome segment in order of increasing molecular mass; the smallest genome segment, then, would be referred to as superhelix (SH) A, and so on. Unfortunately, it has proven impossible to identify all genome segments for any polydnavirus; reasons for this range from inadequacies in gel resolution to comigration of different viral DNAs. Thus, experiments in which genome segments are cloned at random, as here, will almost inevitably identify DNA species additional to those routinely observed following ethidium bromide staining. For this reason, we have used a modification of present practice, in which it is assumed that genome segments additional to those readily visualized within the electrophoretic gel profile exist and will eventually be discovered. Thus, for example, we refer to the smallest genome segment identified here as SH B, which then allows for use of the same system of nomenclature for an additional, smaller genome segment in the event that one is ultimately described; SH B, then, is the smallest superhelix that we have been able to observe, but it may or may not represent the smallest genome segment that exists. Similarly, we have assumed that any relatively large blank zone in the HfPV gel profile may well contain at least one genome segment. Finally, we would also predict that any particularly intensely stained (or broad) DNA band will in fact contain two or more comigrating DNA species; in fact, that has generally been found to be the case. In short, then, we have assigned letters to presently identified HfPV genome segments on the assumption that several additional DNAs will eventually be described. Clearly, such a system cannot accommodate all possible scenarios (e.g., the existence of >26 different viral genome segments), so that some modification of this protocol may in time become necessary.

RESULTS

Molecular cloning of HfPV genome segments. The two-step cloning procedure that we developed during the course of this investigation has allowed us to clone over 30 different HfPV genome segments into the plasmid vector pUC19. Each clone has been named after its parental circle in the HfPV genome, together with the restriction site at which it was cloned; e.g., pHfPV-M (*Hin*dIII) represents the entire viral SH-M cloned into the *Hin*dIII site of pUC19. Most of these clones have been used as probes versus restriction enzyme digests of total viral DNA in order to determine whether (i) restriction fragment length polymorphism was present within the viral genome (14; in each case, virus isolates from at least 12 different female parasitoids were examined) and (ii) cross-hybridization to other HfPV circles could be observed. Among ~30 clones thus far examined, most were found to detect little or no polymorphism within viral DNA (16). As expected from previous work (14), a majority of HfPV DNAs cross-hybridized to one or more additional circles even under conditions of high stringency.

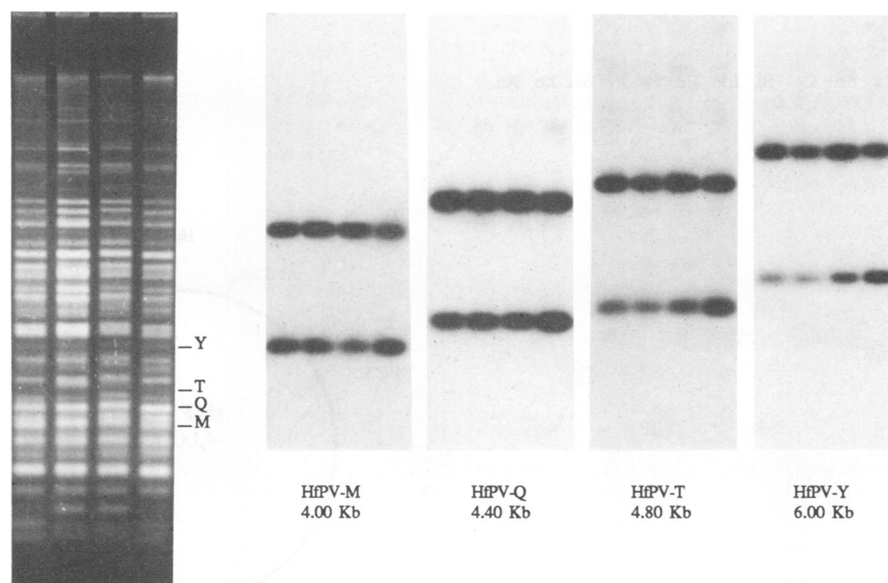

FIG. 1. Cloned genome segments used in this study. On the left, viral DNAs are seen following agarose gel electrophoresis and ethidium bromide staining; viral DNAs from four different female wasps are represented. The positions in the gel of the superhelical forms of genome segments M, Q, T, and Y are indicated. A nylon blot of the same lanes was probed separately with each of the cloned genome segments under conditions of high stringency. In each case, two strong hybridization signals are observed: the lower and upper signals represent, respectively, superhelical and relaxed circular forms of the same molecule. Molecular masses for each genome segment are given.

For our purposes, it was preferable to use as probes only those viral DNAs which exhibited a lack of polymorphism (for the enzymes used) and minimal cross-hybridization. Four cloned genome segments [pHfPV-M(*Hin*dIII), pHfPV-Q(*Kpn*I), pHfPV-T(*Asp*718), and pHfPV-Y(*Sal*I)] acceptably fulfilled these conditions and were therefore regarded as particularly appropriate for a comparative analysis of viral and cognate chromosomal DNAs. In terms of molecular mass, the approximate positions of the corresponding DNA superhelices within the HfPV genome are shown in Fig. 1; in the same figure, viral isolates from four different female parasitoids have been probed with each of the cloned genome segments (plasmid inserts only). In what follows, a relatively detailed analysis of one of the viral DNAs used in this study (HfPV-M) is provided (Fig. 2 to 5) so that the reader can more clearly appreciate the nature and extent of the analysis used throughout; we subsequently provide similar data, but in a more condensed form, for the other three genome segments examined (Fig. 6 to 8).

HfPV-M. (i) Restriction sites. Figure 2 is typical of results obtained by using pHfPV-M as probe versus DNA extracted from virions. Southern blot hybridization (Fig. 2A) and restriction mapping (Fig. 2B) revealed that this genome segment is cut once by *Bam*HI, *Bgl*II, *Eco*RV, *Hin*dIII, and *Pvu*II and twice by *Asp*718 and *Sal*I; the enzymes *Apa*I, *Eco*RI, *Pst*I, *Xba*I, and *Xho*I do not cut SH M. To date, no restriction fragment polymorphism has been detected in viral DNA digested with any of these 12 enzymes; virus isolates from over 50 different individual females have been examined (data not shown). Thus, HfPV-M would appear to be homogeneous within our present *H. fugitivus* colony.

(ii) Site-specific location within the parasitoid genome. To establish that virus-specific DNA may exist in physical (covalent) association with high-molecular-weight (presumably chromosomal) DNA, it is necessary to provide evidence for off-size DNA bands (i.e., bands containing both viral and putative flanking chromosomal sequences) following digestion of genomic DNA with appropriate restriction endonucleases. In the absence of either polymorphism or cross-hybridization, digestion with a zero-cutter (an enzyme that does not cut viral DNA) should generate a single OSF, assuming a single, stable location within the parasitoid genome. Similarly, digestion with enzymes which cut viral DNA one or more times should in each case generate two OSFs, assuming colinearity of viral and cognate parasitoid chromosomal DNA.

From both previous and preliminary work (3, 4, 8), it was assumed that DNA homologous to HfPV genome segments would be detected within the parasitoid genome. In addition, the absence of detectable polymorphism for SH M led us to predict that if any HfPV-specific M sequences were linked to parasitoid genomic DNA, then these too would likely be nonpolymorphic (and would as well probably not possess restriction sites lacking in circular viral DNA). To test these assumptions, male parasitoid DNA was first digested with zero-cutters and then probed with ^{32}P-labelled SH M (insert only from clone pHfPV-M). As shown in Fig. 3A, digests employing *Apa*I, *Eco*RI, *Pst*I, and *Xho*I each generated a single OSF, suggesting that sequences homologous to SH M could be located at a single locus within the wasp genome; these results were in addition suggestive of homogeneity in direct flanking regions. However, in *Xba*I digests, two OSFs were generated; subsequent work (considered below) revealed that this result was due to polymorphism at an *Xba*I site in one of the flanking regions. Consistent with results using zero-cutters, a majority of one- and two-cutters routinely generated two OSFs, in addition to linear fragment(s) of extrachromosomal DNA (Fig. 3B; Table 1). In addition, two one-cutters (*Bgl*II and *Pvu*II) used in combination with zero-cutters typically generated two OSFs (Fig. 3C); as in Fig. 3A, *Xba*I was exceptional, generating a third OSF. Identical results were obtained for the other one-cutters

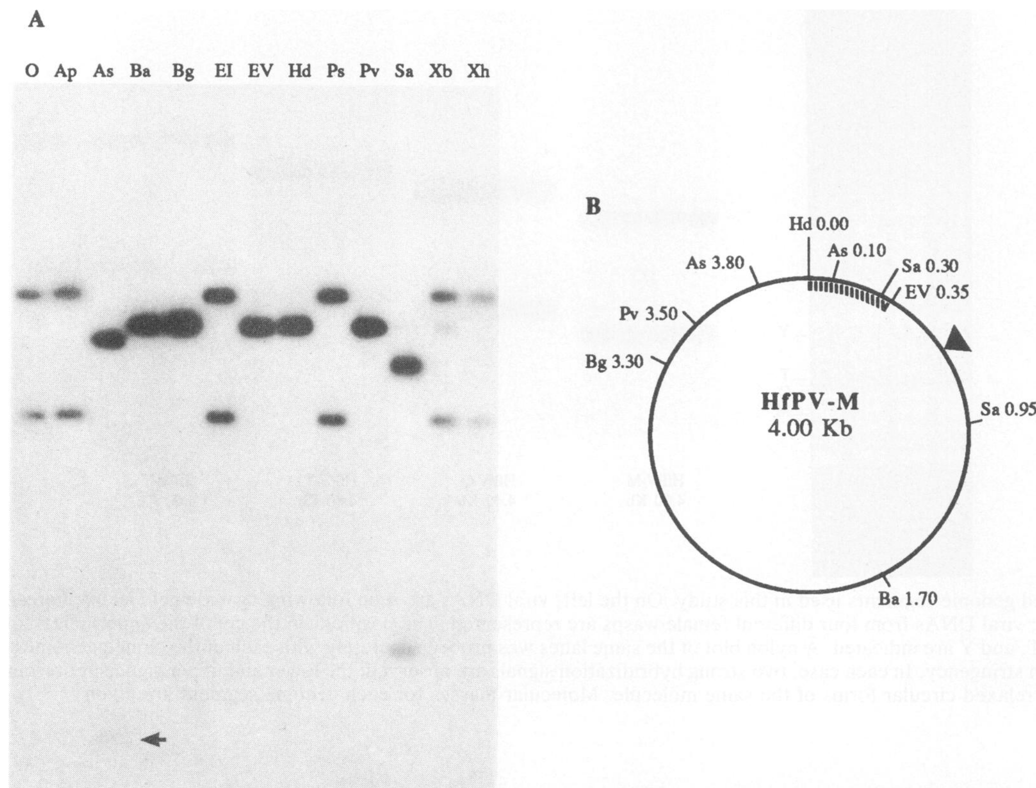

FIG. 2. Southern blot (A) and physical map (B) of SH M; the molecule was cloned into the *Hin*dIII site of pUC19. The blot was probed with the insert only from plasmid pHfPV-M, representing the entire genome segment. The hatched area identifies a 350-bp *Hin*dIII-*Eco*RV fragment used as probe in Fig. 4. The large arrowhead in circular restriction maps, both here and in succeeding figures, denotes the approximate site at which virus-specific DNA within the parasitoid genome is flanked by chromosomal DNA (see also Fig. 5). The very faint 0.30-kb *Asp*718 fragment is indicated (arrowhead). Lanes: O, no enzyme used; Ap, As, Ba, Bg, EI, EV, Hd, Ps, Pv, Sa, Xb, and Xh, restriction enzymes *Apa*I, *Asp*718, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Pvu*II, *Sal*I, *Xba*I, and *Xho*I, respectively (the same designations are used in subsequent figures).

(*Hin*dIII, *Bam*HI, and *Eco*RV; data not shown). Other examples of extra OSFs (three OSFs in the case of *Bam*HI and four for *Sal*I; Fig. 3B) were again traced to the presence of polymorphism in flanking chromosomal domains.

(iii) Restriction site polymorphism in regions flanking chromosomal M-specific DNA. As previously discussed, zero- and one- or two-cutters should generate one and two OSFs, respectively, assuming the existence of a single site for virus-specific sequences within parasitoid genomic DNA and relative stability (i.e., minimal polymorphism) in both virus-specific and flanking chromosomal domains. For the most part, the data obtained for SH M strongly supported these assumptions. The presence of extra OSFs in some digests (e.g., *Xba*I in Fig. 3A and C), however, requires explanation since in the absence of heterogeneity within the M-specific region itself, which was never observed, additional OSFs might in theory suggest that M-specific sequences were located at more than one chromosomal locus (this was, in fact considered rather unlikely, given the weight of evidence in favor of a single such site); alternatively, and more likely, additional OSFs might simply reflect restriction site polymorphism in flanking chromosomal sequences. Since 200 to 300 males were used in preparing genomic parasitoid DNA, it was in our view reasonable to expect that at least some degree of heterogeneity would be observed in these regions. In developing the latter hypothesis, our efforts were facilitated by the use of a small M-specific probe, namely, the 350-bp *Hin*dIII-*Eco*RV fragment, which had previously been mapped to a position near the putative junction site (Fig. 2B); this probe, then, was required in order to define the relative positions of right and left flanking chromosomal domains. The results of this exercise, shown in part in Fig. 4, are summarized in Table 1 (see also Fig. 5). A description of the data presented in Fig. 4 is provided below; these data were used specifically to locate a *Hin*dIII polymorphism (other polymorphisms were identified and analyzed in similar fashion, but raw data are not shown).

Analysis of *Hin*dIII data suggested the presence of a polymorphic site for this enzyme in one of the flanking chromosomal regions. Thus, for example, double digests with *Hin*dIII and zero-cutters routinely generated three OSFs (except for the single case of *Eco*RI) when SH M was used as the probe (Fig. 4A), whereas in the absence of polymorphism only two would have been expected (Fig. 3C). To locate the putative *Hin*dIII polymorphism, the *Hin*dIII-*Eco*RV fragment was used as a probe to define the chromosomal region flanking one side of the M-specific domain (since previous mapping had placed this fragment near the junction site in M DNA). When this probe was used versus the same blot shown in Fig. 4A, only a single OSF was generally observed (except with *Xba*I). In the case of three digests (*Hin*dIII, *Hin*dIII + *Apa*I, and *Hin*dIII + *Xba*I), an OSF was observed at 5.2 kb; three others (*Hin*dIII + *Eco*RI, *Hin*dIII + *Pst*I, and *Hin*dIII + *Xho*I) revealed a

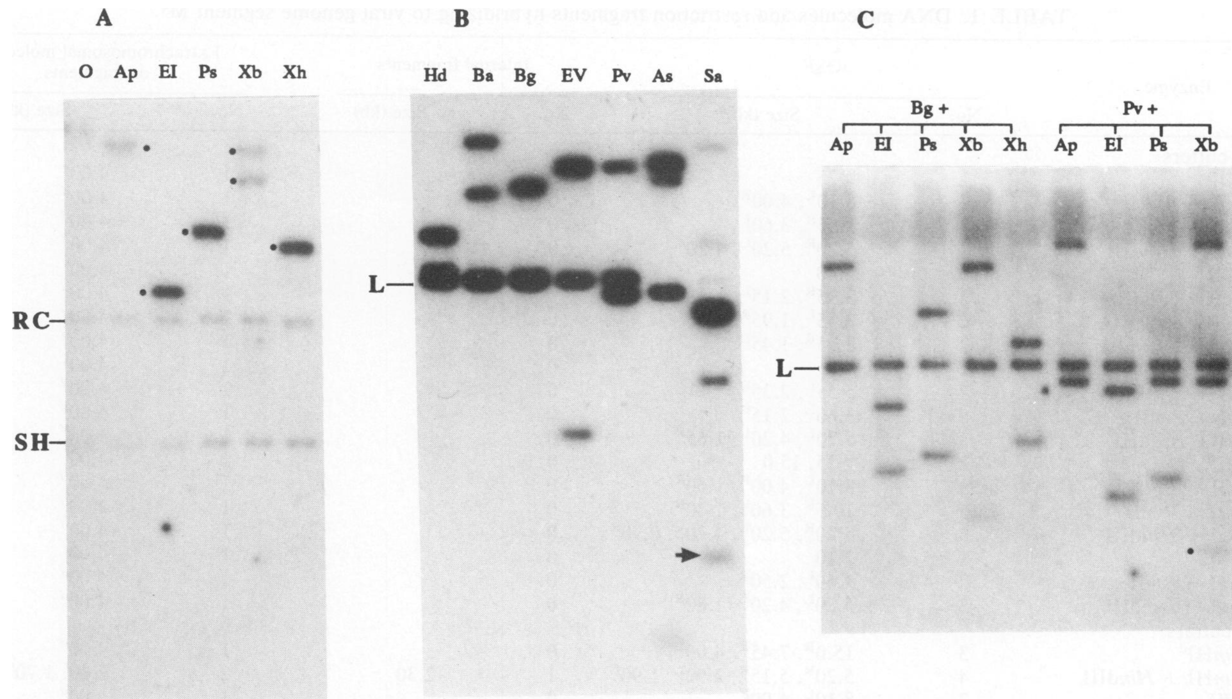

FIG. 3. Southern blots of male genomic DNA digested with various zero- and one-cutters (A and B, respectively) or with the one-cutters *Bgl*II and *Pvu*II, respectively, together with various zero-cutters (C); all blots were probed with pHfPV-M (insert only). OSFs are indicated (dots) for panel A and for the *Pvu*II + *Xba*I lane in panel C; note that *Xba*I generates an additional OSF (panels A and C; see text). The 0.65-kb *Sal*I fragment referred to in the text is identified (arrowhead). SH, RC, and L indicate, both here and in subsequent figures, the positions of superhelical, relaxed circular, and linear forms of extrachromosomal DNA, respectively.

single, relatively small OSF. Again, *Xba*I was atypical, generating two OSFs. The presence of small OSFs suggested that the restriction sites generating them were located near the *Hin*dIII-*Eco*RV region, here arbitrarily defining the right end of the M-specific domain. The data are best explained by assuming the presence of a chromosomal *Hin*dIII site 5.2 kb to the right of the M-specific *Hin*dIII site. The intervening *Xba*I site, then, is polymorphic, while the others examined (*Apa*I, *Eco*RI, *Pst*I, and *Xho*I) are not.

In double digests involving *Hin*dIII and either *Pst*I or *Xho*I (Fig. 4A), OSFs at 5.2 kb were in each case identified when pHfPV-M was used as the probe but not when the 350-bp *Hin*dIII-*Eco*RV fragment was used (Fig. 4B), indicating that particular OSF was derived from chromosomal DNA on the left of the M-specific region; thus, the 5.2-kb band observed in the *Hin*dIII digest probed with SH M contains two comigrating fragments, one from each of the flanking chromosomal regions. An additional OSF at 4.2 kb was also observed in most of the double digests shown in Fig. 4A (but was not detected with use of the 350-bp probe); by analogy with the right-hand *Xba*I site, the two fragments (at 5.2 and 4.2 kb) would appear to represent related OSFs generated by the presence of a polymorphic *Hin*dIII site in the left flanking chromosomal region. The location of these two OSFs was subsequently confirmed by using a 5.0-kb *Hin*dIII-*Pvu*II probe which identifies *Hin*dIII OSFs encompassing chromosomal DNA situated to the left of the M-specific domain (data not shown).

Southern blot hybridization data for SH M together with flanking chromosomal regions are presented in schematic form in Fig. 5. We have in fact already demonstrated the predictive value of this map: the blot shown in Fig. 3C was done after construction of the map; the sizes of the OSFs were precisely as expected.

(iv) Junction site and colinearity with chromosomal DNA. In samples of male genomic DNA, pHfPV-M routinely detected both high-molecular-weight DNA (chromosomal) and smaller (extrachromosomal) species; the latter were detected, in various amounts, in all preparations of male wasp genomic DNA and were observed for all genome segments examined. In the case of zero-cutters, the extrachromosomal DNA always occurred in two forms, and it is assumed here that these represent superhelical and relaxed circular DNAs, since restriction endonuclease digestion of these molecules routinely generated patterns identical to those obtained when authentic viral (i.e., encapsidated) DNA was used. Despite the presence of extrachromosomal circular DNA in parasitoid genomic DNA preparations, we were routinely able to distinguish M-specific fragments generated from chromosomal DNA (i.e., internal M fragments) from those generated from extrachromosomal species, particularly in double-digestion experiments. For example, in *Hin*dIII-*Bam*HI digests, one would of course expect that fragments at 1.70 and 2.30 kb would be generated in the case of viral DNA (see physical map shown in Fig. 2B); the same two bands can be readily identified in a double digest of parasitoid genomic DNA (Fig. 4C), but in this case the larger band is present in excess molar ratio. This finding suggests that the hybridization signal observed at 2.30 kb is derived from both extrachromosomal circles and cognate (M-specific) chromosomal sequences; it follows that the 1.70-kb fragment is not conserved as such within chromosomal DNA, in turn suggesting that M-specific DNA is linked to chromosomal DNA at some site within the 1.70-kb *Hin*dIII-*Bam*HI region.

TABLE 1. DNA molecules and restriction fragments hybridizing to viral genome segment M[a]

Enzyme	OSF		Internal fragments		Extrachromosomal molecules or fragments	
	No.	Size (kb)[b]	No.	Size (kb)	No.	Size (kb)
Zero-cutters						
*Apa*I	1	>25	0		1	4.00[c]
*Apa*I + *Bgl*II	2	8.10[L], 4.00[R]	0		1	4.00[d]
*Apa*I + *Pvu*II	2	10.5[R], 3.60[L]	0		1	4.00[d]
*Apa*I + *Hin*dIII	3	5.20[R], 5.20[L], 4.20[L]	0		1	4.00[d]
*Eco*RI	1	5.40	0		1	4.00[c]
*Eco*RI + *Bgl*II	2	3.35[R], 2.15[L]	0		1	4.00[d]
*Eco*RI + *Pvu*II	2	3.55[L], 1.95[R]	0		1	4.00[d]
*Eco*RI + *Hin*dIII	2	3.95[R], 1.45[L]	0		1	4.00[d]
*Pst*I	1	7.9	0		1	4.00[c]
*Pst*I + *Bgl*II	2	5.55[L], 2.35[R]	0		1	4.00[d]
*Pst*I + *Pvu*II	2	3.60[L], 2.15[R]	0		1	4.00[d]
*Pst*I + *Hin*dIII	3	5.20[L], 4.20[L], 1.65[R]	0		1	4.00[d]
*Xba*I[e]	2	>25, 15.0	0		1	4.00[c]
*Xba*I + *Bgl*II	3	8.10[L], 4.00[R], 1.60[R]	0		1	4.00[d]
*Xba*I + PvuII	3	10.5[R], 3.60[L], 1.40[R]	0		1	4.00[d]
*Xba*I + *Hin*dIII	4	5.20[R], 5.20[L], 4.20[L], 0.90[R]	0		1	4.00[d]
*Xho*I	1	7.10	0		1	4.00[c]
*Xho*I + *Bgl*II	2	4.60[L], 2.50[R]	0		1	4.00[d]
*Xho*I + *Hin*dIII	3	5.20[L], 4.20[L], 1.80[R]	0		1	4.00[d]
One-cutters						
*Bam*HI[e]	3	15.0[R], 7.45[R], 4.00[L]	0		1	4.00[d]
*Bam*HI + *Hin*dIII	4	5.20[R], 5.15[R], 2.90[L], 1.90[L]	1	2.30	2	2.30, 1.70[f]
*Bgl*II	2	8.10[L], 4.00[R]	0		1	4.00[d]
*Bgl*II + *Hin*dIII	3	4.50[L], 3.50[L], 3.30[R]	1	0.70	2	3.30[f], 0.70
*Eco*RV	2	10.0[L], 1.60[R]	0		1	4.00[d]
*Eco*RV + *Hin*dIII	3	5.20[L], 4.20[L], 1.60[R]	1	0.35	2	3.65[f], 0.35
*Hin*dIII[e]	3	5.20[L], 5.20[R], 4.20[L]	0		1	4.00[d]
*Pvu*II	2	10.5[R], 3.60[L]	0		1	4.00[d]
*Pvu*II + *Hin*dIII	2	5.20[R], 3.60[L]	1	0.50	2	3.50[f], 0.50
Two-cutters						
*Asp*718	2	12.0[L], 8.55[R]	1	0.30	2	3.70[f], 0.30
*Asp*718 + *Hin*dIII	3	5.10[R], 5.00[L], 4.00[L]	2	0.20, 0.10	3	3.70[f], 0.20, 0.10
*Sal*I[e]	4	14.0[R], 4.95[R], 3.90[R], 2.15[L]	1	3.35	2	3.35, 0.65[f]
*Sal*I + *Hin*dIII	4	4.90[R], 3.90[R], 2.15[L], 1.15[L]	2	4.05, 0.30	3	4.05, 0.65[f], 0.30

[a] Data derived from pooled male parasitoid DNA digested to completion (Fig. 2 to 4) with the indicated enzymes. One band, *Hin*dIII-*Asp*718, at 0.10 kb, could not be visualized since it ran off the gel. A few other bands (e.g., *Sal*I, 3.90 kb) may be too faint for adequate photographic reproduction. Comigrating bands are observed in some digests at 4.00 or 5.20 kb.
[b] R, OSFs generated from flanking region on the right side as identified by using the *Hin*dIII/*Eco*RV 350-bp fragment as a probe, except 1.60-kb fragments in *Eco*RV and *Eco*RV + *Hin*dIII digests; L, OSFs generated from flanking region on the left side.
[c] Uncut extrachromosomal circle.
[d] Linearized extrachromosomal DNA.
[e] Enzyme identifying polymorphic restriction sites within flanking regions.
[f] Fragment not preserved within chromosomal DNA.

Similarly, the 0.65-kb *Sal*I fragment was underrepresented in digests of chromosomal DNA (Fig. 3B) and so presumably contains the same site. Thus reasoning, we were able to determine the approximate location of putative junction regions on physical maps constructed for all of the genome segments examined in this study.

Insofar as we could determine, restriction sites identified in viral SH M were preserved within M-specific sequences linked to parasitoid chromosomal DNA; similarly, all restriction fragments, except for that encompassing the junction site, were conserved. These observations strongly suggest that extrachromosomal (i.e., viral) and cognate chromosomal M-specific sequences are largely, if not entirely, colinear.

Chromosomal location of HfPV genome segments Q, T, and Y. Data obtained by using three other HfPV genome segments as probes versus male wasp genomic DNA are presented in Fig. 6 to 8. As before, genomic DNA was digested to completion with restriction enzymes known either not to cut a particular viral DNA or else to cut it only once or twice. In each case, only one or two OSFs were observed, depending on the restriction enzyme used. In addition, colinearity with homologous chromosomal DNA was routinely observed. Comparative analysis of genomic and viral DNA digests enabled us to construct schematic diagrams and restriction maps on which putative junction sites between cognate viral and chromosomal DNAs are indicated. As with SH M, restriction polymorphism was not observed in genome segments Q, T, and Y and was also absent in that region of chromosomal DNA homologous to viral DNA. Minor sequence heterogeneity appeared to be present in flanking chromosomal regions, however, as extra OSFs were observed in some digests. Observations relating to Q, T, and Y are briefly summarized below.

Genome segment Q was cloned at the *Kpn*I site into pUC19. Southern hybridization of viral DNA (Fig. 6A) indicated that this genome segment was cut once by *Eco*RI, *Kpn*I, and *Pst*I, twice by *Xho*I, three times by *Hin*dIII, and

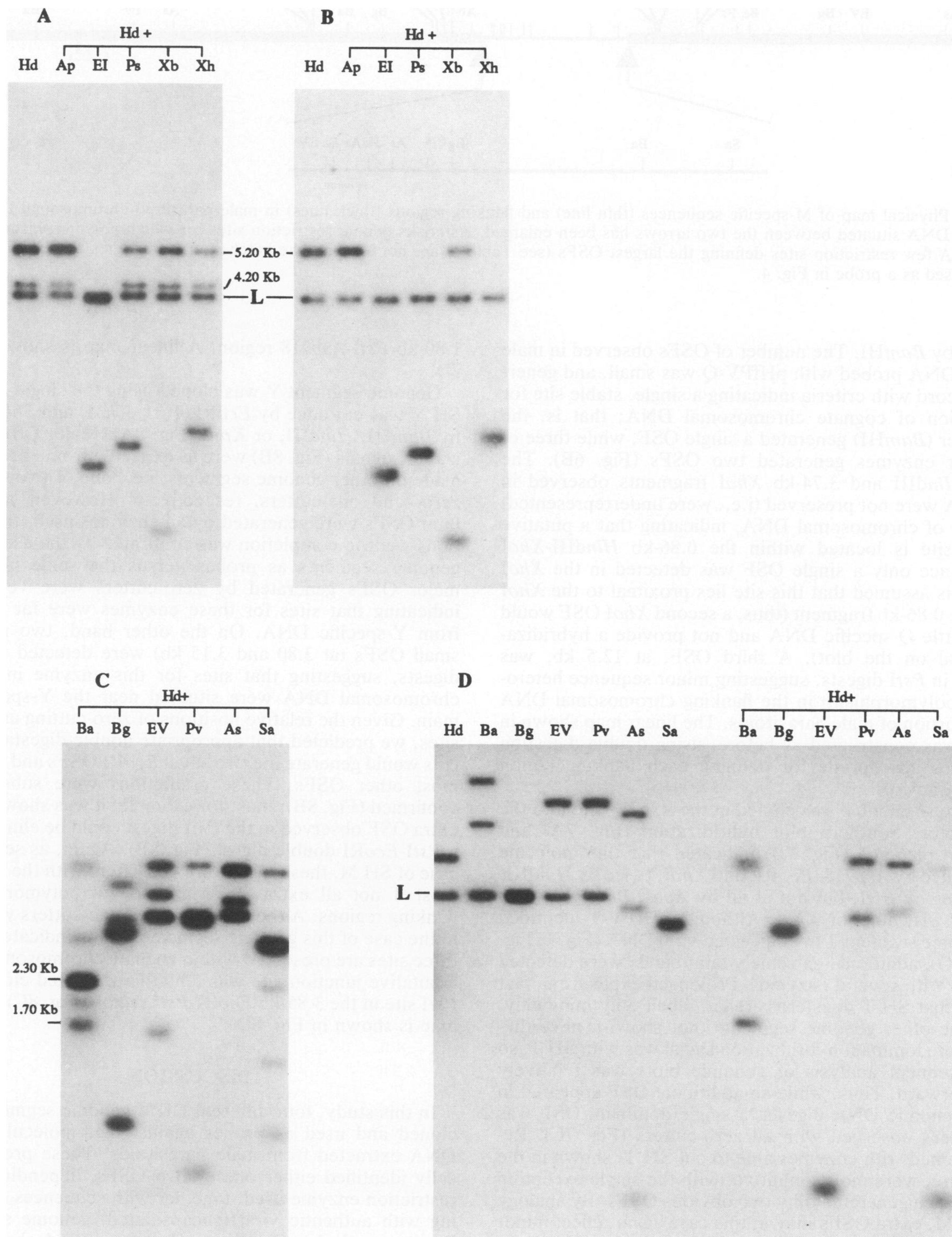

FIG. 4. Southern blots used to construct a detailed physical map of M-specific sequences plus flanking regions in male genomic DNA (see Fig. 5). In most cases, *Hin*dIII has been used in combination with a variety of other enzymes. Blots were probed either with pHfPV-M (A and C) or with the *Hin*dIII-*Eco*RV fragment identified in Fig. 2 (B and D). OSFs discussed in the text are at 5.20 and 4.20 kb in panel A. Extrachromosomal fragments are at 2.30 and 1.70 kb in panel C; note that the 2.30-kb *Hin*dIII-*Bam*HI fragment is in molar excess, indicating that this hybridization signal also contains the cognate internal fragment derived from chromosomal M-specific DNA. Analysis of the data is provided in the text.

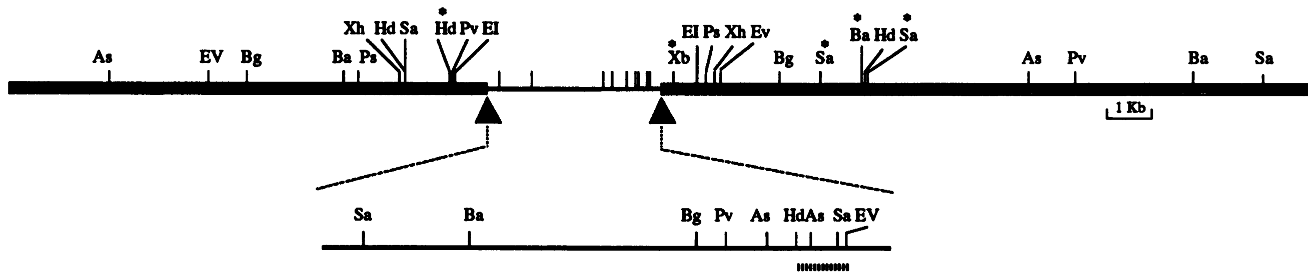

FIG. 5. Physical map of M-specific sequences (thin line) and flanking regions (thick lines) in male parasitoid chromosomal DNA. The M-specific DNA situated between the two arrows has been enlarged. Asterisks denote restriction sites for which polymorphism has been observed. A few restriction sites defining the largest OSFs (see Table 1) are not shown. The hatched area identifies the *Hin*dIII-*Eco*RV fragment used as a probe in Fig. 4.

not at all by *Bam*HI. The number of OSFs observed in male genomic DNA probed with pHfPV-Q was small, and generally in accord with criteria indicating a single, stable site for the location of cognate chromosomal DNA; that is, the zero-cutter (*Bam*HI) generated a single OSF, while three of five other enzymes generated two OSFs (Fig. 6B). The 2.15-kb *Hin*dIII and 3.74-kb *Xho*I fragments observed in viral DNA were not preserved (i.e., were underrepresented) in digests of chromosomal DNA, indicating that a putative junction site is located within the 0.86-kb *Hin*dIII-*Xho*I region; since only a single OSF was detected in the *Xho*I digest, it is assumed that this site lies proximal to the *Xho*I end of the 0.86-kb fragment (thus, a second *Xho*I OSF would contain little Q-specific DNA and not provide a hybridization signal on the blot). A third OSF, at 12.5 kb, was observed in *Pst*I digests, suggesting minor sequence heterogeneity (polymorphism) in the flanking chromosomal DNA of a proportion of male parasitoids. The linear map shown in Fig. 6D was constructed as before, using double digestion and probes appropriate for defining each flanking region (data not shown).

Genome segment T was cloned at the *Asp*718 site into the same vector. Southern blot hybridization (Fig. 7A) and restriction mapping (Fig. 7B) indicated that this molecule was cut once by *Asp*718, *Eco*RI, and *Xho*I, twice by *Hin*dIII, three times by *Pst*I, but not at all by *Apa*I, *Bam*HI, *Bgl*II, *Eco*RI, *Pvu*II, *Sal*I, or *Xba*I. Although pHfPV-T identified only genome segment T in undigested viral DNA (Fig. 1; Fig. 7A, lane O), additional extremely faint bands were detected in digests with several enzymes. Prolonged exposure in fact revealed that SH T does cross-react, albeit only minimally, with some other genome segments (not shown); nevertheless, the predominant hybridization signal was with SH T, so that subsequent analysis of genomic blots was relatively straightforward. Thus, while an additional OSF appeared in several genomic DNA digests, a single dominant OSF was nevertheless observed with all zero-cutters (Fig. 7C). Results obtained with enzymes able to cut SH T, shown in the same figure, were more definitive: with the single exception of *Xho*I, each generated only two obvious OSFs. By analogy with SH M, extra OSFs may in this case again reflect minor sequence variability in flanking chromosomal DNA. As observed for SH M and Y, chromosomal T-specific and viral DNAs were also colinear, since restriction sites and internal fragments (e.g., the 1.80-kb *Hin*dIII fragment and the 1.35- and 0.95-kb *Pst*I fragments) were well preserved in digests of genomic DNA (not shown). Southern blot experiments utilizing different regions of SH T as probes (not shown) confirmed the existence of a junction site located within the 1.80-kb *Pst*I-*Asp*718 region. A linear map is shown in Fig. 7D.

Genome segment Y was cloned using the single *Sal*I site. SH Y was cut once by *Eco*RI, *Pst*I, *Xho*I, and *Sal*I but not by *Bam*HI, *Hin*dIII, or *Kpn*I (Fig. 8A). Major OSF hybridization signals (Fig. 8B) were as expected from observations made on other genome segments, i.e., one or two OSFs for zero- and one-cutters, respectively. However, additional faint OSFs were generated by all enzymes used (that digestions went to completion was confirmed by the use of other genome segments as probes versus the same blot). The major OSFs generated by zero-cutters were very large, indicating that sites for these enzymes were far removed from Y-specific DNA. On the other hand, two relatively small OSFs (at 3.80 and 3.15 kb) were detected in *Eco*RI digests, suggesting that sites for this enzyme in flanking chromosomal DNA were situated near the Y-specific domain. Given the relative positions of zero-cutting and *Eco*RI sites, we predicted that appropriate double-digestion protocols would generate the two small *Eco*RI OSFs and eliminate most other OSFs. These predictions were subsequently confirmed (Fig. 8B); thus, for example, it was shown that an extra OSF observed in the *Pst*I digest could be eliminated in a *Pst*I-*Eco*RI double digest (Fig. 8B). Again, as seen in the case of SH M, these results are consistent with the view that most if not all extra OSFs are due to polymorphism in flanking regions. Although only three one-cutters were used in the case of this genome segment, results indicated that all three sites are preserved within cognate chromosomal DNA; a putative junction site was tentatively located close to the *Pst*I site in the 3.85-kb *Eco*RI-*Pst*I region (Fig. 8C). A linear map is shown in Fig. 8D.

DISCUSSION

In this study, four different HfPV genome segments were cloned and used as probes against high-molecular-weight DNA extracted from male parasitoids. These probes typically identified either one or two OSFs, depending on the restriction enzyme used, together with sequences comigrating with authentic viral (encapsidated) genome segments. Recent work by Fleming and Summers (4) has clearly established that the OSFs detected in blots of *C. sonorensis* DNA are indicative of a physical linkage between cognate CsPV and flanking parasitoid genomic DNAs, and there is likewise little reason to doubt that the OSFs observed here in *H. fugitivus* DNA constitute presumptive evidence for a chromosomal location for HfPV DNA. In ongoing studies, we have now examined almost 30 different HfPV genome

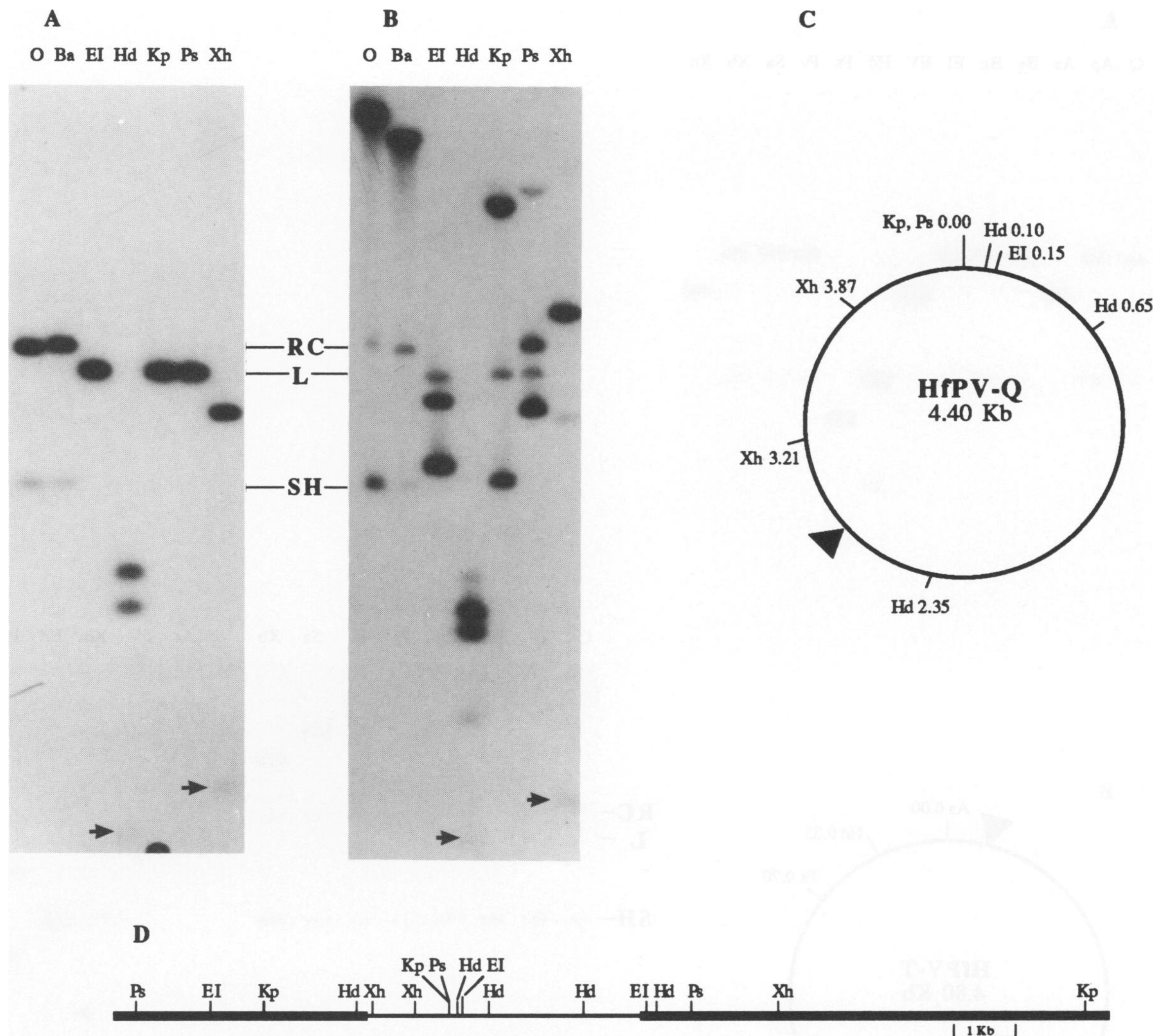

FIG. 6. SH Q. (A and B) Southern blots of viral and genomic DNA, respectively, probed with the entire genome segment; (C and D) physical maps of viral and cognate genomic DNAs, respectively. The positions of two faint restriction fragments at 0.55 kb (*Hin*dIII) and 0.66 kb (*Xho*I) are indicated for viral DNA in panel A and for extrachromosomal DNA in panel B (arrowheads). The positions of superhelical (SH), relaxed circular (RC) and linear (L) forms of the extrachromosomal DNA are indicated; additional hybridization signals in panel B represent OSFs (except lane O, which represents undigested DNA).

segments and have observed OSFs for each within parasitoid DNA. As with the four genome segments examined here, all appear to be situated at unique chromosomal loci; only one (SH S) was found to have two cognate chromosomal sites (16). We believe that a majority of the HfPV genome has now been examined, and we extrapolate from this to conclude that sequences homologous to the entire HfPV genome likely have a chromosomal location. In a forthcoming report, we examine the location within the parasitoid genome of those HfPV genome segments which cross-hybridize strongly with others. Preliminary data clearly establish the existence of several different families consisting of two or more such genome segments; each genome segment family appears to be situated at a single chromosomal locus (16).

It now becomes relevant to ask whether a chromosomal location for polydnavirus genomes may represent a general phenomenon. On this question, the available evidence, while limited, nevertheless seems compelling. In the case of the two most carefully studied examples, namely CsPV and HfPV (3, 4; this study), essentially identical observations have now been recorded, and it is of interest that these two viruses share little, if any, obvious sequence homology (5). Preliminary work on a third ichnovirus, from *Hyposoter lymantriae*, also provides clear evidence for a chromosomally located polydnavirus genome (16). In addition, we have shown that sequences homologous to a bracovirus genome (from *Cotesia melanoscela*) are located within the parasitoid genome (16). It thus seems reasonable to suggest that a chromosomal location for polydnavirus genomes may represent a fundamental characteristic defining this unusual virus family.

A comparison of restriction maps generated for encapsidated viral and cognate chromosomal DNAs indicates that these are largely, if not entirely, colinear; similar data have been reported for CsPV (3, 4). Such observations have important implications. For example, colinearity would tend to suggest that virus-specific chromosomal loci are maintained with a remarkable degree of stability within the

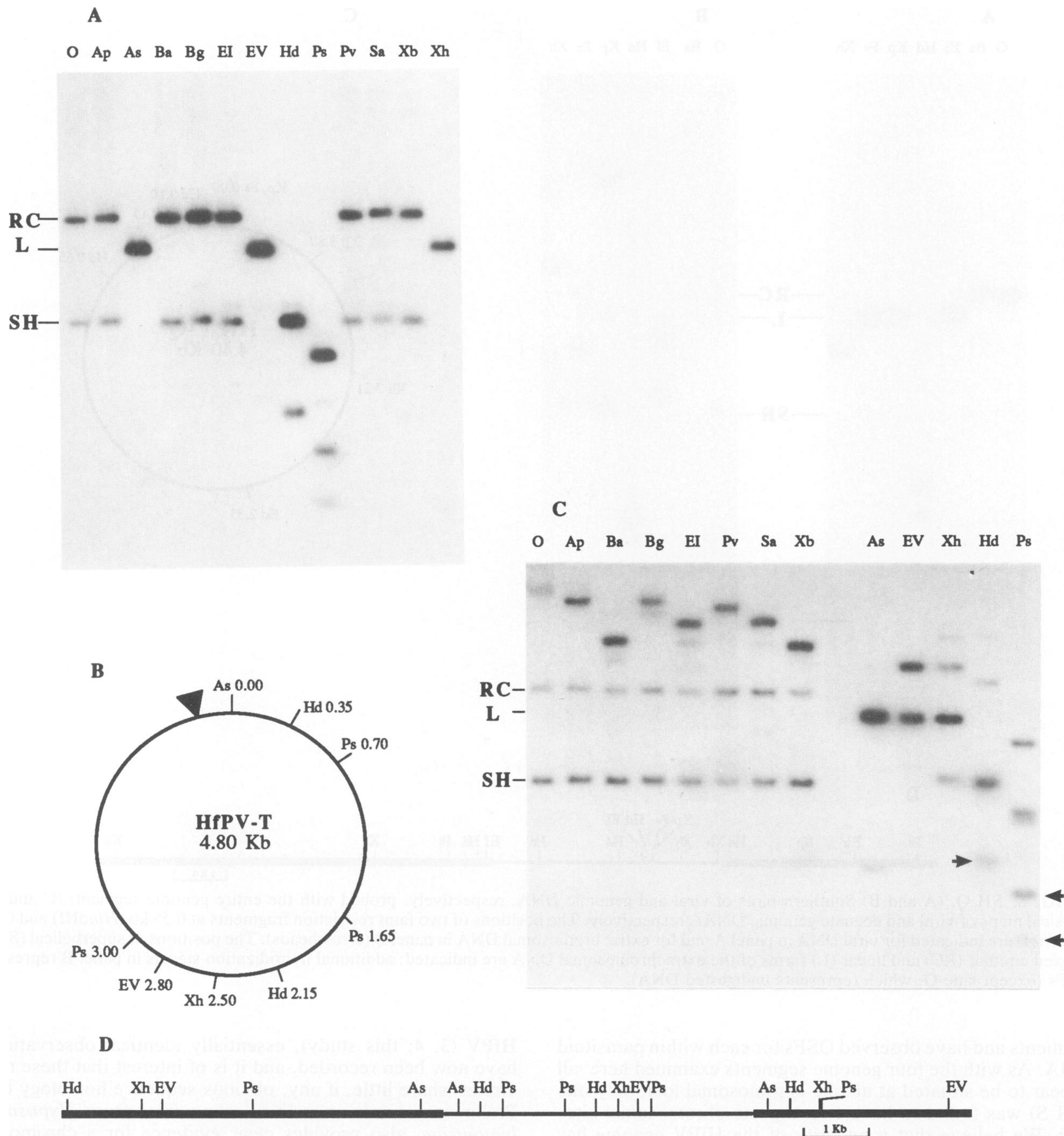

FIG. 7. SH T. (A) Southern blot of viral DNA probed with the entire genome segment; (B) physical map; (C) male genomic DNA digests probed with SH T. Note that a single predominant OSF is generated in genomic digests using zero-cutters (lanes Ap to Xb), but some very faint OSFs are also observed. Most of the latter were eliminated by digestion with enzymes cutting SH T (C, lanes As to Ps). Fragments internal to the T-specific domain in genomic DNA digests are indicated by arrowheads at 1.8 kb (*Hin*dIII) and 1.35 and 0.95 kb (*Pst*I). (D) Map of chromosomal T-specific and flanking domains.

parasitoid genome; the observation that chromosomal sequences homologous to any given viral genome segment are typically located at only a single site also supports this argument. It seems to us unlikely that precision of this order would obtain were it not of functional significance. In that regard, we can suggest one obvious possibility: a chromosomal template is required for the replication of polydnavirus DNA, a requirement in fact predicted by earlier work on the chromosomal transmission of polydnavirus genomes (8).

We have given considerable attention in this study to the question of polymorphism. Polydnavirus genomes are both large and complex, and they may therefore be expected to be at least some extent polymorphic (14). We note, however, that both viral and cognate chromosomal DNAs are pre-

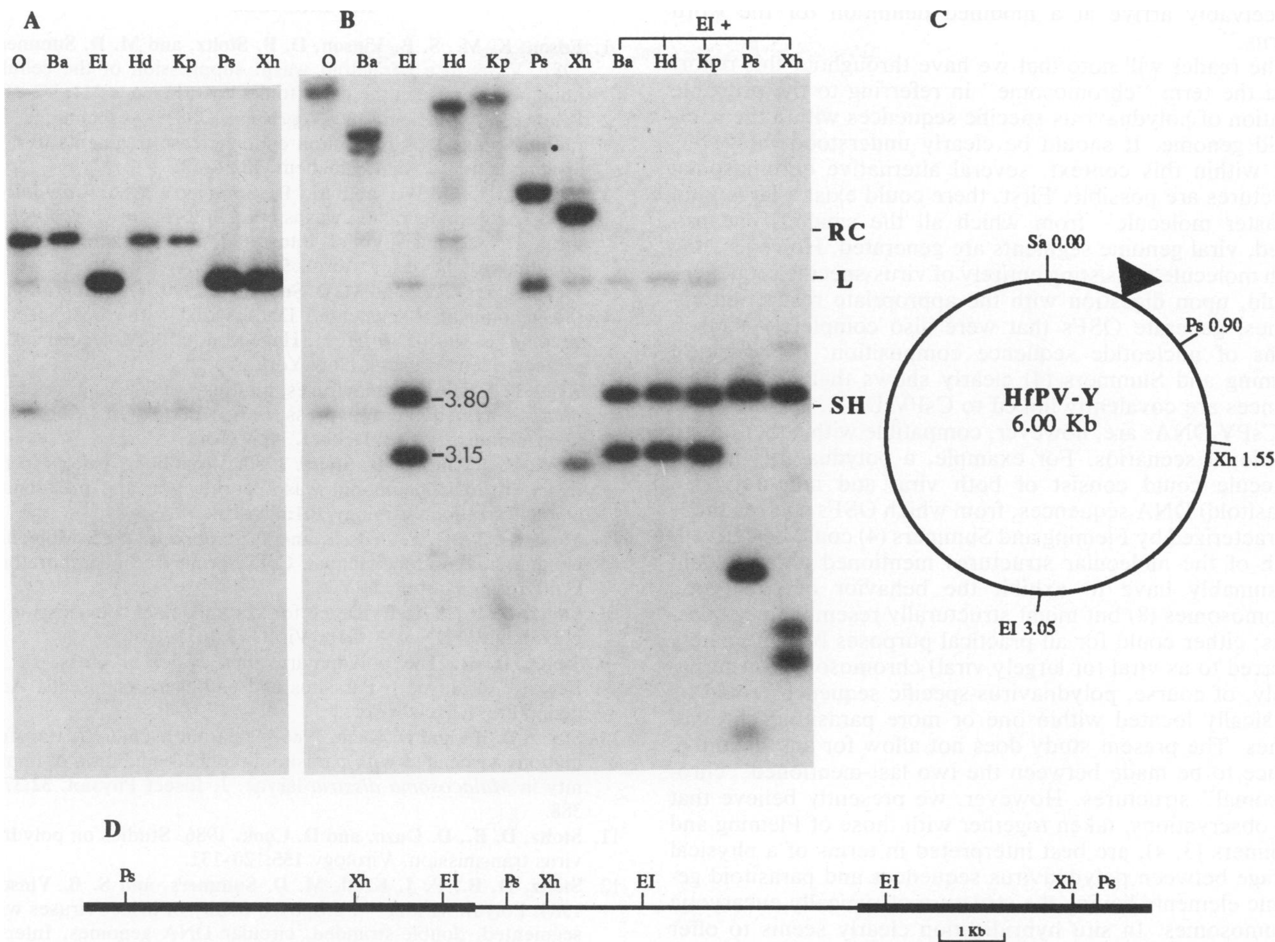

FIG. 8. SH Y. (A and B) Southern blots of viral and genomic DNA, respectively, probed with the entire genome segment; (C and D) physical maps of viral and cognate genomic DNAs, respectively. The 3.80- and 3.15-kb *Eco*RI OSFs referred to in the text are indicated in panel B; in the same blot, note that the indicated extra *Pst*I OSF is eliminated in the *Eco*RI-*Pst*I double digest.

cisely equivalent in terms of restriction site polymorphisms: where absent in the former, they were also found to be absent in the latter; where present, they appear in both (16). These observations again support the view that encapsidated viral genome segments are in some manner derived, ultimately, from a chromosomal template (8). Needless to say, polymorphism may also be expected to occur in flanking chromosomal regions as well as in virus-specific DNA. The problem here is that polymorphism in flanking regions needs to be distinguished from the alternate possibility of multiple chromosomal loci for sequences cognate to any given viral genome segment, since either scenario would produce extra OSFs in genomic blots. As we have shown, extra OSFs can usually be eliminated by careful planning, which includes a judicious choice of restriction enzymes; our work suggests that the presence of extra OSFs in *H. fugitivus* genomic digests will most often result from polymorphism in one or more of the flanking chromosomal domains. In fact, the degree of polymorphism which we have observed may be considered to be relatively minor, considering the noninbred nature of our present parasitoid colony; this supports the conclusion, made both here and elsewhere (3, 4), that sequences homologous to polydnavirus genome segments are stably located within parasitoid chromosomal DNA.

The stable, site-specific location of polydnavirus DNA within insect genomes is unique in virology. While many other viruses may integrate some or all of their genomes into chromosomal DNA, unique chromosomal loci are very rarely involved. More importantly, other chromosomally located viral genomes are observed in only a fraction of the host population, whereas polydnavirus genomes are present in all individuals of every affected species; this is best explained by assuming that endogenous polydnavirus sequences were acquired prior to the speciation of those parasitoids which carry them. It may now, perhaps, be instructive to begin thinking of the polydnavirus life cycle as comprising two distinct arms, or pathways: in one, a linear chromosomal provirus is responsible for transmission and, presumably, replication (8); in the other, a circular encapsidated form of the viral genome directs a genetic colonization of the parasitized host, thereby ensuring survival of the developing parasitoid (9). The polydnavirus phenomenon inevitably forces us to reconsider the extent of possible virus-host interactions; in so doing, we could

conceivably arrive at a modified definition for the word "virus."

The reader will note that we have throughout this report used the term "chromosome" in referring to the probable location of polydnavirus-specific sequences within the parasitoid genome. It should be clearly understood, however, that within this context, several alternative chromosomal structures are possible. First, there could exist a large viral "master molecule" from which all the smaller, encapsidated, viral genome segments are generated. However, any such molecule consisting entirely of virus-specific sequences would, upon digestion with the appropriate restriction enzymes, generate OSFs that were also completely viral in terms of nucleotide sequence composition; the work of Fleming and Summers (4) clearly shows that nonviral sequences are covalently linked to CsPV DNA. Observations on CsPV DNAs are, however, compatible with either of two additional scenarios. For example, a polydnavirus master molecule could consist of both viral and nonviral (i.e., parasitoid) DNA sequences, from which OSFs such as those characterized by Fleming and Summers (4) could be derived. Both of the molecular structures mentioned above would presumably have to exhibit the behavior of eucaryotic chromosomes (8) but might structurally resemble megaplasmids; either could for all practical purposes be legitimately referred to as viral (or largely viral) chromosomes. Alternatively, of course, polydnavirus-specific sequences could be physically located within one or more parasitoid chromosomes. The present study does not allow for any definitive choice to be made between the two last-mentioned "chromosomal" structures. However, we presently believe that our observations, taken together with those of Fleming and Summers (3, 4), are best interpreted in terms of a physical linkage between polydnavirus sequences and parasitoid genomic elements having the structure of typically eucaryotic chromosomes. In situ hybridization clearly seems to offer the best approach to resolving the issue; this work is currently in progress.

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