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Recombinant baculoviruses as vectors for identifying proteins encoded by intron-containing members of complex multigene families

(*Bombyx mori* nuclear polyhedrosis virus/silk moth chorion proteins/RNA splicing/mRNA translation/posttranslational processing)

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ABSTRACT **Using a transfer vector derived from *Bombyx mori* nuclear polyhedrosis virus (BmNPV), we have constructed recombinant baculoviruses that contain complete silk moth chorion chromosomal genes encoding high-cysteine proteins under the control of the polyhedrin promoter. Silk moth tissue culture cells infected with these recombinant viruses were found to contain abundant RNA sequences of sizes similar to those of the authentic chorion mRNAs. Chorion transcripts present in infected cells were initiated almost exclusively at the cap site of the polyhedrin start site. Primer extension and RNase protection experiments revealed that a considerable proportion of the resultant transcripts were spliced at the same sites as those utilized in follicular cells for the production of functional chorion mRNA. Electrophoretic analysis and immunoprecipitation of the proteins of host cells infected with the recombinant viruses revealed the presence of the corresponding chorion proteins. We conclude that baculovirus vectors can be used for expressing efficiently not only cDNAs or simple genes devoid of intervening sequences but also intron-containing chromosomal genes. Thus, recombinant baculoviruses offer a powerful alternative to hybrid-selected translation, particularly when the identification of proteins encoded by members of complex multigene families is required.**

Nuclear polyhedrosis viruses or class A baculoviruses represent a group of DNA viruses that attack a variety of insect species, particularly lepidoptera. Recently these viruses have received considerable attention because of their potential as vectors for expressing foreign DNA of various origins in insect cells (1, 2). A key feature of these vectors is the utilization of a powerful promoter element that controls the expression of the gene encoding the major late viral protein, polyhedrin, the predominant constituent of the characteristic occlusion bodies or polyhedra that accumulate in the nuclei of infected cells during the late stages of infection. Foreign genes placed under the control of the polyhedrin promoter are transcribed at high rates by insect cells infected with the corresponding recombinant viruses, and large quantities of the encoded polypeptides are synthesized in these cells near the end of the infection cycle. Proteins synthesized in insect cells have been shown to undergo a variety of intracellular posttranslational modifications, and in many cases the modifications have resulted in the acquisition of biologically active products.

Thus far, only a single intron-containing gene, the early transcription unit of simian virus 40 (SV40) encoding the small and large tumor antigens (t and T antigens, respectively), has been expressed under polyhedrin promoter control (3, 4). SV40 transcripts synthesized in infected cells were spliced predominantly at the t antigen splice junction. Transcripts spliced at the T antigen-specific junction were grossly underrepresented. Immunoprecipitations of the proteins of the infected cells showed the presence of small amounts of t antigen and, to a much lesser extent, of T antigen as well.

In this study, we have explored further the possibility of expressing chromosomal genes under the control of the polyhedrin promoter. We have reasoned that, if successful, such a system would be particularly useful in characterizing multigene families, where identification of specific gene products by other currently available methods, such as hybrid selection (5, 6), is very difficult because of cross-hybridization of homologous mRNA sequences to the gene fragments of interest. We have used a vector system based on the polyhedrin promoter of *Bombyx mori* nuclear polyhedrosis virus (BmNPV) (7, 8) and tested it with two silk moth chorion chromosomal genes belonging to two multigene families, *HcA* and *HcB*, each of which comprises 15 closely related members (9, 10). We demonstrate that silk moth cells infected with each of the two recombinant BmNPVs are capable of transcribing efficiently the polyhedrin promoter-driven chorion genes, that posttranscriptional processing of the chorion transcripts occurs correctly and efficiently, and that the encoded polypeptides are synthesized and processed posttranslationally in the infected cells to yield the corresponding mature chorion proteins.

MATERIALS AND METHODS

Cells and Viruses. *B. mori* Bm5 tissue culture cells (11) were maintained and infected with BmNPV as described (12). Recombinant BmNPVs were plaque-purified following cotransfection of Bm5 cells with mixtures of DNA from recombinant transfer vectors and wild-type BmNPV (12) as described (7).

Transfer Vectors, Chorion Clones, and Probes. BmNPV transfer vectors pBmp and pBmp2 used for generating recombinant viruses BmNPV/HcA.12, BmNPV/HcB.12, and BmNPV2/HcA.12-HcB.12 have been described (7). Transfer vector pBmp/HcA.12 contained gene *HcA.12* as a 1.55-kilobase (kb) *Dde* I fragment derived from sc4.150 (13); vector pBmp/HcB.12 contained gene *HcB.12* as a 733-base-pair (bp) *Hin*cII insert isolated from sc13.150 (13); and vector pBmp2/HcA.12-HcB.12 contained the complete *HcA.12-HcB.12* gene pair as a 3.75-kb *Eco*RI fragment isolated from sc4.150. All fragments were inserted into the unique *Xba* I sites of the transfer vectors by blunt-end ligation. For primer extension from the first exons of the two chorion genes, 5′-end-labeled oligonucleotides complemen-

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Abbreviations: BmNPV, *Bombyx mori* nuclear polyhedrosis virus; t and T antigens, small and large tumor antigens, respectively; nt, nucleotides.

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tary to the sense strands of the genes were used (residues +35 to +61 for gene *HcA.12* and +29 to +56 for gene *HcB.12*). The same oligonucleotides were also used as hybridization probes. For primer extension from the second exon of gene *HcB.12*, a double-stranded fragment comprising residues +168 to +381 and labeled at the 5′ end of the antisense strand was used. The labeled fragment was obtained by end-labeling a 2.4-kb *Ava* I fragment of sc13.150 and subsequently digesting with Bsp1286.

Primer Extensions. Primer extensions were carried out as described (14) with 2 μg of total RNA and 50,000 cpm Cerenkov of primer. Template/primer annealing was at melting temperature (t_m) +3°C (for the double-stranded primer) or at t_m −15°C (for the oligomer primers). Extension products were analyzed on wedge-spaced (0.2–0.6 mm) 6% polyacrylamide/8 M urea sequencing gels (15).

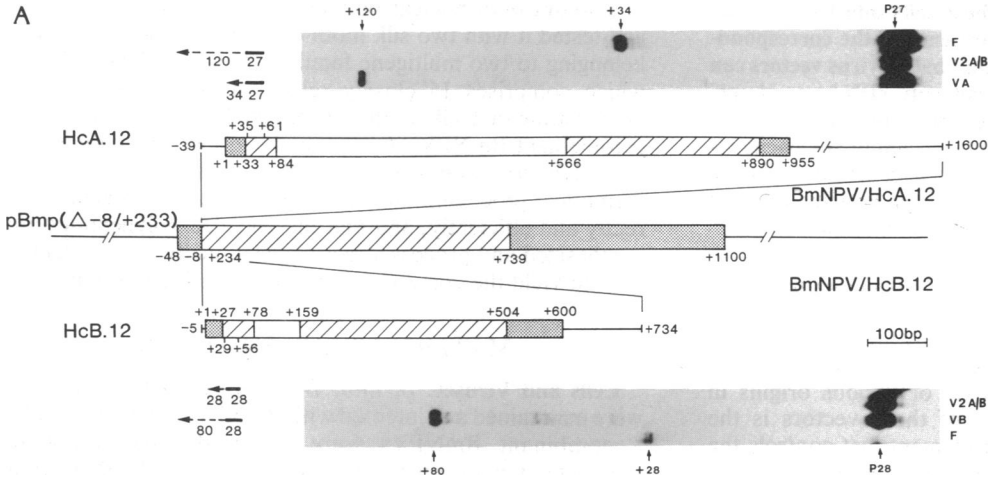
Riboprobe Synthesis and RNase Protection. Subclone pHcB(0.5) (a pGEM-1 derivative; Y. A. W. Skeiky and K.I., unpublished data), containing a 0.5-kb *Ava* II fragment of gene *HcB.12*, was transcribed with phage T7 RNA polymerase after linearization with *Xba* I. RNase protection mapping was carried out as described (16) after overnight annealing of 5 μg of total RNA with 200,000 cpm Cerenkov of [α-^{32}P]CTP-labeled riboprobe.

RNA Blot-Hybridization (Northern) Analysis. Conditions for RNA isolation, electrophoretic analysis on methylmercuric hydroxide-agarose gels, transfer to hybridization membranes, and hybridization were as described (14).

Protein Analysis. Bm5 cells were labeled for 6 hr prior to harvesting with 100 μCi of L-[^{35}S]cysteine (Amersham; 600 Ci/mmol, 10 mCi/ml; 1 Ci = 37 GBq). Labeled cells were solubilized with 8 M urea/0.36 M Tris·HCl, pH 8.4/50 mM dithiothreitol at a rate of 10^4 cells per μl of solution, and proteins were carboxamidomethylated by incubation with iodoacetamide (17). Immunoprecipitations were carried out on aliquots of carboxamidomethylated proteins containing 0.5–3.5 × 10^5 cpm diluted to 500 μl with 150 mM Tris·HCl, pH 7.5/0.05% Nonidet P-40. The protein solution was preadsorbed for 1 hr at room temperature with 3 μl of preimmune rabbit serum; after precipitation with a 30-μl suspension of *Staphylococcus aureus* protein A (The Enzyme Center, Malden, MA) for 30 min, supernatant proteins were immunoprecipitated with 3–4 μl of serum from rabbits immunized with a preparation of total carboxamidomethylated chorion proteins and 30–40 μl of the protein-A suspension. Precipitated immune complexes were washed with Tris/Nonidet P-40 buffer, resuspended in 20 μl of protein electrophoresis buffer, and released into the supernatant by boiling for 5 min. Immunoprecipitated proteins were analyzed by SDS/polyacrylamide gel electrophoresis (17) or two-dimensional isoelectric focusing–SDS/polyacrylamide gel electrophoresis (18).

RESULTS

Bm5 Cells Infected with BmNPV/HcA.12 and BmNPV/HcB.12 Contain Mature-Size Chorion Transcripts Initiated at the Polyhedrin Gene Start Site. The structures of the transfer vectors used for acquiring the two chorion recombinant



B

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                                        -48
                                       ┌-->
---GGAAATAATAACCATCTCGCAAATAAATAAGTATTTTACTGTTTTCGTAACAGT

                  -8    (-39)
TTTGTAATAAAAAAACCCTCTAGTTTTACTATATAAGAACGAAGTCTTGAAGCTTAA

 (+1)                                MetPheThrPhe   CysCysC
AGTATTCAAGATTAAGTGCCTGAATATCCAGCATCATGTTTACCTTCXYZTGTTGCT

ysTer              (+1600)  +234
GCTAAZWTCATAGACCCGTCTAACTAGAGTGAAACCCGATACCATGAAGTTAAT---

                                        -48
                                       ┌-->
---GGAAATAATAACCATCTCGCAAATAAATAAGTATTTTACTGTTTTCGTAACAGTTTT

            -8    (-5) (+1)                          MetAlaLys
GTAATAAAAAAACCCTCTAGAACTCATTCTGAAGTCATTCAGTGAAACAACATGGCCGCT

Lys   GlyCysCysTER    (+729)   +234
AAAXYZGGTTGCTGCTAAZWTGTGTTCTAGAGTGAAACCCGATACCATGAAGTTAAT---
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FIG. 1. (*A*) Construct maps and primer extensions. Chorion genes *HcA.12* and *HcB.12* were inserted into transfer vector pBmp2 [pBmp-(Δ−8/+233)], and the resultant plasmids were used for obtaining viruses BmNPV/HcA.12 and BmNPV/HcB.12. Untranslated gene regions are stippled, coding regions are hatched, introns are open, and flanking sequences are indicated by lines. The results of the primer extension assays are shown above and below the maps. Predicted extensions of the synthetic primers (solid lines) are shown by broken arrows. On the autoradiograms, P indicates the primers. Lanes F, VA, VB, and V2A/B contain extension products obtained from RNA of control follicular cells and Bm5 cells infected with BmNPV/HcA.12, BmNPV/HcB.12, and BmNPV2/HcA.12-B.12, respectively. Arrows indicate the predominant extension products. (*B*) Sequence details for BmNPV/HcA.12 (*B Upper*) and BmNPV/HcB.12 (*B Lower*) at the insert junctions. Chorion gene sequences are underlined. Numbers and numbers in parentheses are those of the polyhedrin and chorion genes, respectively. Junction sequences are boxed. Bold letters X, Y, Z, and W indicate the first exon, intron, second exon, and 3′ flanking region of the inserts.

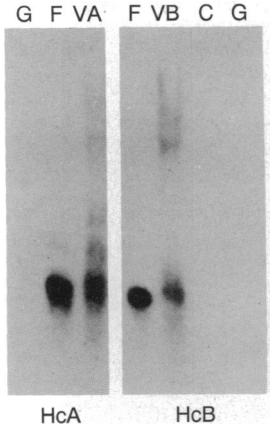

FIG. 2. Northern hybridizations. RNA extracted from follicular cells of wild-type strain 703 (lanes F), mutant strain Gr^B lacking all *Hc* genes (lanes G), uninfected control Bm5 cells (lane C), and Bm5 cells infected with BmNPV/HcA.12 (lane VA) or BmNPV/HcB.12 (lane VB) was resolved on a denaturing agarose gel and, after transfer, probed for the presence of HcA (*Left*) and HcB mRNA (*Right*) sequences by hybridization to the corresponding oligonucleotides. The predominant hybridizing RNA species in cells infected with each of the two chorion recombinant viruses has a mobility similar to authentic HcA.12 or HcB.12 mRNA.

baculoviruses are shown in Fig. 1*A*. The chorion gene fragments, comprising sequences from −39 to +955 (gene *HcA.12*; cap site at +1) and from −5 to +729 (gene *HcB.12*) were inserted adjacent to residue −8 of the polyhedrin gene of BmNPV (cap site at −48 according to the baculovirus convention, which defines the adenosine of the initiator methionine codon as +1).

The polyhedrin promoter-directed transcriptional expression of the two chorion genes in infected Bm5 cells was examined first by primer extension analysis. As shown by the results of the electrophoretic analysis presented in Fig. 1*A*, >95% of the chorion transcripts that could be detected by the two primers were found to be initiated at the polyhedrin mRNA start site (see Fig. 1*B* for sequence details).

A further analysis of the RNA of infected cells was carried out through Northern hybridizations (Fig. 2). RNA of uninfected Bm5 cells and follicular cell RNA derived from a wild-type and a mutant silk moth strain, Gr^B, which lacks all *Hc* genes (19, 20), was similarly analyzed. The majority of chorion transcripts in the infected cells (lanes VA and VB) had sizes similar to those of authentic HcA.12 and HcB.12 mRNA of wild-type follicular cells (lanes F). No hybridization was obtained with control Bm5 cell RNA (lane C) or follicular RNA of the Gr^B mutant strain (lanes G).

The combined results of the experiments presented in Figs. 1 and 2 suggest that the chorion polyadenylylation signals are efficiently utilized and that intron splicing is also occurring in the infected cells. This is particularly evident for gene *HcA.12*, which contains a 482-bp intron sequence (10) that, if present, should result in a hybridizing species almost twice as long as mature HcA.12 mRNA.

Polyhedrin Promoter-Derived Transcripts Are Correctly Spliced. Because from the results presented above we could not be certain whether correct splicing of HcB.12 transcripts, which contain a short intron (Fig. 1*A*), occurs in the infected cells, we characterized further these transcripts by a combination of primer extension and RNase protection experiments. A 214-bp singly end-labeled fragment derived from the second exon of the *HcB.12* gene (Fig. 3*A*) was used as primer in the extension experiments. This primer should be extended by 219 nucleotides (nt) on correctly initiated but unspliced transcripts or by 138 nt on correctly initiated and spliced chorion mRNA (Fig. 3*B*).

The analysis of the extension products derived from the RNA of BmNPV/HcB.12-infected cells was facilitated by comparisons to those obtained from several control RNA preparations. Primer extension using wild-type follicular cell RNA (Fig. 3*B*, lane F) resulted in the expected 86-nt extension of HcB.12 mRNA (initiation at +1; Fig. 3*A*). This extension was absent from the extension products of RNA of follicular cells of the mutant silk moth strain Gr^B (Fig. 3*B*, lane B), which lacks all Hc mRNAs. The bands that were observed with Gr^B RNA, probably represent nonspecific

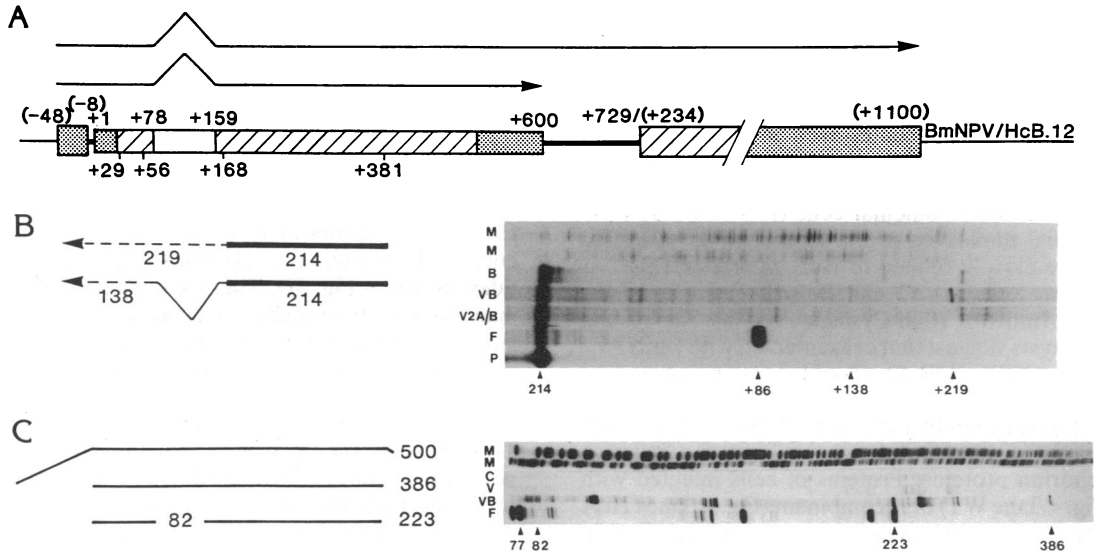

FIG. 3. RNA splicing in cells infected with BmNPV/HcB.12. (*A*) Map of the region of insertion of gene *HcB.12* (thin boxes) into the polyhedrin gene (thick boxes). All gene conventions are as in Fig. 1. Arrows indicate transcripts initiating at the polyhedrin cap site and terminating at the polyhedrin and chorion polyadenylylation sites. (*B*) Primer extension assays. (*B Left*) The labeled primer (thick solid line) and predicted extensions (broken arrows) on spliced (thin solid line) and unspliced transcripts initiated at the polyhedrin start site. Note that extension products obtained with authentic HcB.12 mRNA should be 52 nt shorter than those derived from transcripts initiated at the polyhedrin start site (the length of the polyhedrin leader and linker sequences; Fig. 1). (*B Right*) Primer extension results. Lane designations indicate the origin of RNA templates: F, wild-type follicular cells; B, Gr^B mutant follicular cells; VB, Bm5 cells infected with BmNPV/HcB.12; V2A/B, Bm5 cells infected with BmNPV2/HcA.12-HcB.12; P, the unextended primer; and M, chemical DNA sequencing ladders. (*C*) RNase A and T1 protection assays. (*C Left*) Labeled riboprobe and predicted protected fragments from unspliced and correctly spliced chorion transcripts initiated at the polyhedrin start site. Note that the riboprobe fragment protected by the first exon of authentic HcB.12 mRNA should be 5 nt shorter than the corresponding species from the recombinant virus (the length of the 5′ untranslated region of the chorion gene present in the riboprobe; see Fig. 1). (*C Right*) Fragments protected from RNase cleavage. Lanes: V, RNase protection patterns of Bm5 cells infected with wild-type BmNPV; C, RNase protection patterns from uninfected control Bm5 cell. All other lane designations are as in *B*.

extensions from cross-hybridizing follicular RNA sequences. Primer extension with RNA from Bm5 cells infected with another recombinant virus, BmNPV2/HcA.12-HcB.12 (Fig. 3*B*, lane V2A/B), which contains the two chorion genes as a single insert in a domain that is devoid of polyhedrin promoter activity (see ref. 7 for details on this construct), resulted in several extension products. These included all the nonspecific extensions detected with Gr^B mutant follicular RNA and some additional ones that are due to the presence of randomly initiated HcB.12 transcripts in these cells (K.I. and R.G.M., unpublished data). Finally, the electrophoretic analysis of the extension products obtained with the RNA of cells infected with BmNPV/HcB.12 (Fig. 3*B*, lane VB) revealed the presence of only two new products (arrows)—the predicted 219- and 138-nt extensions, corresponding to the unspliced and spliced chorion RNAs transcribed under the control of the polyhedrin promoter (Fig. 3*A*). In this experiment, ≈25% of the correctly initiated chorion transcripts appear to be spliced correctly. It is worth mentioning that we have observed an inverse correlation between stage of infection and proportion of correctly spliced transcripts. Correctly spliced HcB.12 transcripts were more abundant at 39 hr than at 56 hr after infection or longer (data not shown, but see chorion protein section below).

The results of the primer extension assays were extended by a RNase protection analysis using a 500-nt riboprobe that was partially complementary to the HcB.12 sequence transcribed from BmNPV/HcB.12 (Fig. 3*C*). Annealing of the riboprobe to correctly initiated primary transcripts of infected cells should result in the protection of a 386-nt fragment, while annealing to correctly spliced mRNA should result in two protected fragments, 82 nt from the first exon (77 nucleotides in the case of authentic HcB.12 mRNA of follicular cells) and 223 nt from the second exon. Both types of protected fragments were obtained with RNA from cells infected for 56 hr (Fig. 3*C*, lane VB). Additional protected species were also evident in the autoradiogram. The cluster of protected fragments that are longer than the second exon are probably due to RNase "nibbling" occurring in the A+T-rich intron region (10) of the riboprobe–unspliced transcript duplexes. This is corroborated by the decreased ratio of unspliced to spliced molecules seen in this experiment relative to that revealed through the primer extension analysis (Fig. 3*B*). The multiplicity of protected fragments in the reaction with RNA from follicular cells (Fig. 3*C*, lane F) is due to the partial duplexing of the riboprobe to other Hc mRNAs that are closely related to HcB.12 mRNA (21).

Correctly Processed HcA.12 and HcB.12 Transcripts Are Translated into Authentic High-Cysteine Proteins. The results of the RNA analysis suggest that cells infected with BmNPV/HcA.12 and BmNPV/HcB.12 should contain substantial quantities of functional chorion mRNA that may be translated into the corresponding chorion proteins. Therefore, these cells were examined immunologically for the presence of the two chorion proteins. Proteins of cells infected with wild-type (Fig. 4, lane WT) or recombinant (Fig. 4, lanes HcA and HcB) BmNPVs for 39 hr and 56 hr were analyzed first on an SDS gel. One predominant (arrow) and a few minor immunoprecipitable species were found in the cells infected with each of the two recombinant viruses. Interestingly, cells infected for 56 hr or longer were consistently found to contain fewer immunoprecipitable polypeptides than cells infected for 39 hr or less, in contrast to the situation observed with the accumulation of polyhedrin in cells infected with wild-type BmNPV. The "satellite" polypeptides migrating just above the main ones may represent precursor chorion polypeptides containing their 21-amino acid signal peptide sequence (22) or, alternatively, modified polypeptides (23). For the HcB.12 protein, the two alternatives can be distinguished by two-dimensional electrophoresis because its signal peptide contains a charged lysine residue (10) (the signal peptide of HcA.12 lacks charged residues).

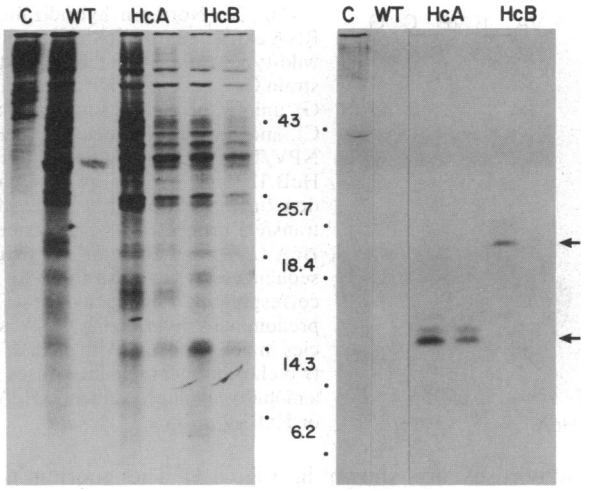

FIG. 4. Protein analysis. Total (*Left*) or immunoprecipitated (*Right*) labeled proteins were analyzed by SDS/polyacrylamide gel electrophoresis. Lanes: C, uninfected control Bm5 cells; WT, cells infected with wild-type BmNPV; HcA, cells infected with BmNPV/HcA.12; HcB, cells infected with BmNPV/HcB.12. For each pair of lanes, the left one contains proteins from cells infected for 39 hr, while the right one contains proteins of cells collected 56 hr after infection. The single polypeptide in the right WT lane of *Left* is the major late viral protein, polyhedrin. The positions of the main immunoprecipitable chorion polypeptide species are indicated by arrows in *Right*. Note that Hc chorion proteins migrate with an abnormal mobility relative to molecular weight size markers in kb, whose position is indicated by dots, because of their unusual amino acid composition (≈30% cysteine residues) and the carboxamidomethylation treatment.

Comparisons of the two-dimensional profiles of the labeled proteins obtained from cells infected for 39 hr with wild-type BmNPV (Fig. 5*A*) and BmNPV/HcB.12 (Fig. 5*B*) revealed that the latter contain a prominent set of polypeptides that is absent from wild-type BmNPV-infected cells. As shown in Fig. 5*C*, this set of polypeptides was immunoprecipitable and migrated with a mobility similar to that of a set of two authentic high-cysteine chorion proteins of strain 703 (Fig. 5*D*, circled proteins) from which gene *HcB.12* was derived. On the basis of this analysis, we conclude that gene *HcB.12* encodes Hc protein c.6 (24), the smaller of the two polypeptides circled in Fig. 5*D*. The streaking nature of the predominant immunoprecipitable species may be due to a posttranslational modification resulting in an intramolecular structuring of the polypeptide similar to that recently reported to occur in follicular cells (23). It is also clear that all of the major immunoprecipitable species have the same isoelectric point. This excludes the possibility that they represent precursor polypeptides or degraded products occurring inside the cells or during handling. Finally, the minor immunoprecipitable products of higher molecular weight and more acidic isoelectric points (Fig. 5*C*) were also present in uninfected control cells and, therefore, appear to be immunologically cross-reacting nonchorion species.

Two-dimensional analysis of the proteins of BmNPV/HcA.12-infected cells (not shown) revealed that the encoded polypeptide barely enters the first dimension (pH 4). Its identification tentatively places it at the position of the previously identified chorion protein a.3 (24) or at a more basic position.

DISCUSSION

Our results have shown that, under appropriate conditions, efficient expression of a protein can be obtained from an

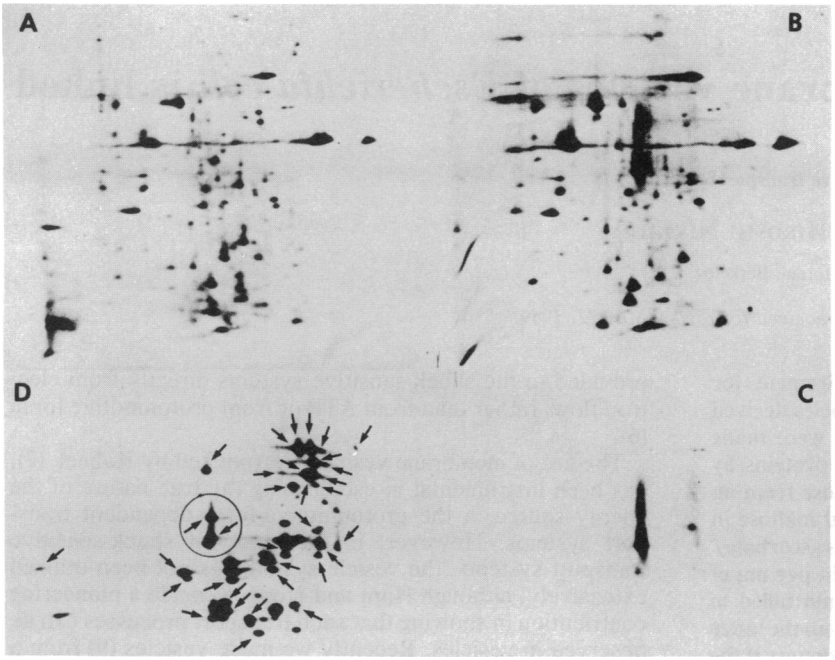

FIG. 5. Two-dimensional separation of labeled proteins. First dimension (left to right) is isoelectric focusing (basic end at left), and second dimension (top to bottom) is SDS gel electrophoresis. (*A*) Proteins from Bm5 cells infected with wild-type BmNPV. (*B*) Proteins of cells infected with BmNPV/HcB.12 for 39 hr. (*C*) Immunoprecipitable proteins from cells infected as in *B*. (*D*) Authentic chorion proteins of silk moth strain 703, from which gene *HcB.12* was derived. Arrows indicate the chorion polypeptides eliminated by the Gr^B deletion (24), which encompasses all *Hc* and a number of *HcA* and *HcB* chorion genes (19, 20). The two chorion polypeptides comigrating with the immunoprecipitable polypeptides for BmNPV/HcB.12 cells are circled.

authentic, intron-containing gene placed under the control of the polyhedrin promoter. Our detailed analyses of chorion RNA transcripts accumulating in Bm5 cells infected with the recombinant BmNPVs have established that, even at the late stages of infection, infected cells maintain their basic ability to undertake correctly all the steps of the mRNA biosynthetic pathway, including splicing.

Our finding that Bm5 cells infected for a period of 39 hr or less contain more immunoprecipitable polypeptides than cells infected for 56 hr or longer (Fig. 5 and data not shown) merits special attention because it suggests that the splicing machinery of the cells may be gradually incapacitated at the late stages of viral infection. A more systematic analysis is obviously needed for obtaining a firm correlation between relative and absolute accumulation of correctly spliced transcripts as a function of the duration of infection.

Although we do not know whether this system will be as efficient in processing transcripts from complex chromosomal genes of other organisms, the successful, albeit low, expression of simian virus 40 t and T antigens in insect cells under polyhedrin promoter control (3, 4) suggests that baculovirus-mediated expression of chromosomal genes should be of general applicability. Current methods of identification of gene products are mainly based on hybrid selection of specific mRNAs or on *in vitro* synthesis of RNA from available cDNA clones coupled to translation in cell-free systems or in *Xenopus* oocytes. For genes belonging to multigene families, hybridization-based mRNA selection results in the isolation of multiple cross-reacting species and in an equivalent number of translated polypeptides (see refs. 5 and 25 for earlier results on the chorion system). In contrast, expression of chromosomal structural genes under polyhedrin promoter control results in the exclusive generation of the authentic encoded polypeptides in their mature form. Finally, the feasibility of obtaining authentic gene products from chromosomal genes placed under polyhedrin promoter control obviates the need for generating full-length cDNA clones for protein identification purposes.

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