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Localization of Domains within the *Drosophila* Ref(2)P Protein Involved in the Intracellular Control of Sigma Rhabdovirus Multiplication

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The *ref(2)P* gene of *Drosophila melanogaster* interferes with sigma rhabdovirus multiplication. This gene is highly variable, and the different alleles are considered permissive or restrictive according to their effects on virus replication. In all cases, the mechanisms involve intracellular interactions between the sigma virus and Ref(2)P proteins. We showed that the N-terminal domain of the Ref(2)P protein was required for its activity in vivo. The protein was inactive in the null p^{od2} mutant when its first 82 amino acids were deleted. The $p^{\Delta n}$ gene was constructed so that the first 91 amino acids coded for by the restrictive alleles could be expressed in vivo. It was active in a transformed line. This sequence was sufficient to impart a restrictive phenotype to an adult *D. melanogaster* fly after it was injected with the virus. However, the truncated protein expressed by $p^{\Delta n}$ did not have an effect on the hereditary transmission of the sigma virus to the offspring of the infected flies, even though it contained the restriction site. The native Ref(2)P protein has been previously shown to have conformation-dependent epitopes common with some of those of the viral N protein. We demonstrated the following. (i) These epitopes were found in a domain of the Ref(2)P protein distinct from the site involved in restriction. (ii) They were modified in the N protein of the *haP7* sigma virus mutant selected as being adapted to the restrictive alleles of the *ref(2)P* gene; only one mutation in the N gene, leading to an amino acid substitution, distinguished the *haP7* mutant from the original virus. (iii) The virus strains partially or totally adapted to the effects of the full restrictive protein expressed by p^{r} were always found to multiply to a lesser extent in the presence of the protein expressed by $p^{\Delta n}$. These data suggest that two distinct domains of the Ref(2)P protein are involved in the control of sigma virus multiplication.

The expression of cellular genes can selectively control the outcome of viral infections. Sigma rhabdovirus infection of *Drosophila melanogaster* can be studied as a model system in which the restriction of viral activity by host defense mechanisms allows for the survival of both the host and the virus. A portion of the natural *D. melanogaster* population is infected with the sigma virus, which survives only by hereditary transmission to the progeny of infected flies and which cannot invade an insect population by horizontal transmission. It maintains a persistent infection in the cytoplasm of both somatic and germinal cells without any apparent cytopathogenic effects (3). The infected flies are fatally sensitive to high concentrations of CO_2, whereas uninfected flies survive such exposures. This effect essentially allows the virus to be experimentally selected and studied (5, 8).

Several host genes involved in resistance to infection have been identified by genetic analysis. The *ref(2)P* gene, the most extensively studied of these genes, is highly polymorphic in natural *Drosophila* populations, and this variability has been confirmed by S1 nuclease mapping and sequencing of different haplotypes (10). The numerous alleles can be classified as permissive or restrictive by comparing their effects on sigma virus multiplication. The restrictive alleles limit the replication of certain virus strains. These effects can be studied when the conditions of a primary infection are experimentally reproduced by injection of the virus into uninfected adult flies. Measurement of the extent to which the virus can multiply, invade the organism, and establish a persistent infection allows distinctions to be made among the different *ref(2)P* alleles. The probability of infection reaches 100% in a permissive context but falls to 0.01% for viral strains sensitive to the restrictive alleles (12). When a healthy female crosses with an infected male, the virus infects some of the progeny of a permissive female, but it is not transmitted or is only poorly transmitted to the offspring of a female with a restrictive genotype. The hereditary transmission of sigma virus from persistently infected female flies can also be altered by the genotype of the progeny. Permissive females supply the virus to all embryos; however, in the presence of restrictive alleles, virus multiplication is strongly reduced in the somatic tissues and in the germ line and the virus cannot be detected in the progeny (14).

Several characteristics strongly suggest that direct interactions occur among the products of *ref(2)P* and sigma virus genes. (i) The *ref(2)P* gene product acts specifically and exclusively on the sigma virus (2). (ii) The effects of the restrictive alleles vary widely on the basis of the virus strain (12). (iii) Analysis of the virus mutants shows that a single mutation is sufficient for them to escape the control of the *ref(2)P* gene product. Many of these mutants are also found to be temperature sensitive (4, 5). Thus, viral proteins would be involved in the interactions with the cellular gene products. (iv) The interactions involve both the permissive and the restrictive alleles, which are codominant, and their products compete with one another to act on the virus. In a heterozygous strain, the presence of a permissive allele counteracts the activity of a restrictive one (16). (v) A dosage effect on virus replication can

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be seen in the number of the restrictive *ref(2)P* gene copies expressed at the locus (16). Protein-protein interactions have been observed at the molecular level. The Ref(2)P protein is found in complexes associated with the viral P protein, which is required for RNA polymerase activity. Furthermore, the cellular protein shares conformation-dependent epitopes that are common with some of those of the structural N protein associated with the viral genome (21).

Null mutations of the *ref(2)P* gene have been experimentally obtained after mutagenesis of the permissive *ref(2)p^o* allele (*p^o*) (6, 13). The products of these mutants lost their function in the virus cycle. Unlike the products of the original permissive allele, these mutant products cannot compete with those of the restrictive *ref(2)P^P* allele (*p^p*) and their activity decreases in heterozygous *p^p/p^{null}* strains. Nonetheless, the null alleles are completely permissive for sigma virus multiplication in homozygous *p^{null}/p^{null}* strains. These data show that the product of a permissive allele is not necessary for virus replication, although it interacts with the viral proteins. Thus, the *ref(2)P* gene product is involved in the control of infection only in its restrictive form. The homozygous *ref(2)P^{null} Drosophila* strains are viable. The *ref(2)P* gene is not essential at the cellular level, but males are sterile in particular genetic backgrounds.

We studied null mutations, including nonsense mutations and a deleted *ref(2)P* gene constructed in vitro and expressed after transformation. An analysis of their properties enabled us to distinguish between the domains of the Ref(2)P protein containing the restrictivity site and the epitopes common with the viral N protein.

MATERIALS AND METHODS

***D. melanogaster* and virus strains.** The wild-type alleles, permissive *ref(2)p^o* and restrictive *ref(2)p^p* and *ref(2)p^n* (*p^o*, *p^p*, and *p^n*, respectively), have already been described (10). The null *ref(2)p^{od1}*, *ref(2)p^{od2}*, and *ref(2)p^{od3}* mutations (*p^{od1}*, *p^{od2}*, and *p^{od3}*) were obtained with the *p^o* allele by mutagenesis with diepoxybutane (13). To obtain a hemizygous genotype at the *ref(2)P* locus (*p/def*), we used the Df(2L)TWE55 chromosome in which the *ref(2)P* gene is totally deleted (13). The 23DA and A3 sigma viruses are natural strains sensitive to the restrictive *ref(2)P* alleles (12). The *haP7* and 23SP$^+$ mutants were selected in the 23DA virus to grow in the presence of the restrictive *p^p* allele (5).

Gene construction and transformation. The internal *Nar*I-*Nar*I region corresponding to the sequence for the majority of exon 2, intron 2, and a part of exon 3 was deleted from the cloned *Hin*dIII-*Xho*I genomic DNA fragment containing the *p^p* allele. The resulting fragment, containing the allele called *p$^{\Delta n}$*, encoded the first 91 amino acids (aa) of the restrictive protein, including 57 aa from exon 1 and 34 aa from the beginning of exon 2, plus a glycine residue at the C-terminal end. Expression of the *ref(2)P* gene was analyzed after intron 2 was deleted as follows. The *Nar*I-*Nar*I genomic DNA fragment was replaced by the *Nar*I-*Nar*I fragment isolated from the cDNA of the restrictive *p^n* allele. The modified *p$^{n\text{-}i2}$* gene, in the absence of intron 2, encoded the full-length Ref(2)P protein. The *p$^{\Delta n}$* and *p$^{n\text{-}i2}$* constructions were cloned into the pW6 vector. This plasmid contains a polylinker and the *white Drosophila* gene inserted between the inverted terminal repeats of the P transposon (15). Embryos carrying the *white* mutation in chromosome 1 and the stable Δ2-3 P element in chromosome 3 as a transposase source were microinjected with the recombinant DNA clones to establish transformed lines (17, 18). Both the *white* gene and either the *p$^{\Delta n}$* or the *p$^{n\text{-}i2}$* allele were inserted into the *Drosophila* genome by transposition. The progeny of the adult flies which had lost the *white* phenotype as a result of transformation were selected for the crossings appropriate to eliminate the chromosome carrying the Δ2-3 P element and to control the resident *ref(2)P* locus.

Sigma virus assay. Only sigma virus-infected flies were paralyzed by and died after a 15-min exposure to pure CO_2. This characteristic was used to measure the infection rates in the two types of experiments analyzing the effects of the *ref(2)P* alleles on sigma virus multiplication. First, the virus was injected into adult flies. The probability of initiating a primary infection on the basis of the genotype was compared with that for a reference strain. Second, the virus was vertically transmitted from persistently infected females to their progeny. The frequencies of CO_2-sensitive flies among offspring having different genotypes were compared. The assay methods and calculations have been described previously (8, 16).

RNA purification and dot-blotting analysis. Total RNAs were prepared from embryos and adult flies, and poly(A)$^+$ RNAs were selected on oligo(dT) cellulose columns as previously described (6). Purified RNAs were denatured in 1 M glyoxal and 10 mM phosphate buffer (pH 6.5) for 1 h at 50°C. Samples were diluted at least 10-fold in the phosphate buffer, and increasing mRNA amounts were dotted onto a GeneScreen membrane. Filters were baked for 4 h at 80°C. Dot blots were hybridized with nick-translated DNA probes consisting of the restriction fragments from the genomic *ref(2)P* DNA containing 75% of the mRNA 5′ end. Hybridizations were performed as previously described (6). After autoradiography, the individual dots were quantitated to evaluate the proportions of the *ref(2)P* mRNA found in the poly(A)$^+$ RNAs. The actin messenger was used as an internal reference in similar dot-blotting experiments by hybridizing it with a 1.8-kb fragment of an actin gene cloned into the pDM4B1 plasmid (a gift of J. L. Couderc) (7).

Immunoprecipitation and Western blot (immunoblot) analysis. The preparation and purification of anti-Ref(2)P protein antibodies have already been described (21). T-REF antibodies were raised against the whole permissive Ref(2)P protein expressed in recombinant baculovirus-infected *Spodoptera frugiperda* cells. Sigma virus ribonucleoprotein (RNP) antibodies were directed against the proteins associated with the viral RNP complex. The immunoprecipitation of the proteins has been described previously (21). Under denaturing conditions, the proteins were extracted from ground embryos or adult flies in 1× SB (62 mM Tris-HCl [pH 6.8], 1% sodium dodecyl sulfate [SDS], and 1.25% β-mercaptoethanol) and a mixture of protease inhibitors called CLAPA (1 μg [each] of chymostatin, leupeptin, antipain, and pepstatin per ml and 8 μg of aprotinin per ml) at 100°C for 15 min. Debris was separated out by two centrifugations at 12,000 × *g* for 20 min each time. The supernatants were diluted 10-fold with 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and 1% sodium deoxycholate and were incubated overnight at 4°C with 2 to 10 μl of purified antibodies per ml. Under native conditions, embryos and adult flies were ground and suspended in 10 mM Tris-HCl (pH 7.4), 10 mM KCl, 0.15 mM $MgCl_2$, and CLAPA for 30 min at 4°C, homogenized by 30 strokes with a Dounce homogenizer, and centrifuged twice at 12,000 × *g* for 20 min each time. The supernatants were adjusted to 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.1% Triton X-100 before incubation with 10 μl of purified antibodies per ml. Immune complexes were collected with protein A-Sepharose beads, and the proteins were analyzed by Western immunoblotting. Signals were amplified with a streptavidin system and were revealed by detection of the horseradish peroxidase activity with a light-based enhanced chemiluminescence system (Amersham). To compare the protein amounts accumulated in the wild and mutant strains, the Ref(2)P protein was immunoprecipitated from a set of dilutions of each protein extract, prepared under denaturing conditions, and analyzed by Western blotting as described above. The amounts detected were compared with those in the *p^o* adult flies, taking into account the number of gene copies at the locus and the sizes of the polypeptides. This comparison was possible because we verified that the T-REF antibodies, which were always added in excess, allowed almost equal detection of the different regions of the Ref(2)P protein (data not shown).

Nucleotide sequence analysis. Poly(A)$^+$ RNAs were purified from *p^o* adult flies infected with the 23DA or *haP7* virus as described above. A synthetic oligonucleotide, 5′ GCCGGATCCTTTTTTTTTTTTCATGAGCTGTCA 3′, was used as a primer for the 3′ end in the following two reactions: (i) the reverse transcription of the mRNA of the N protein and (ii) the subsequent PCR amplification in association with an oligonucleotide (5′ GCCGTCGACTTTAC TATGGAACAAGCCAAACTC 3′) complementary to the 5′ end. The nucleotide sequence was obtained by the dideoxy chain terminator method (T_7 sequencing kit; Amersham). The oligonucleotide sequence was deduced from the published sequence of the sigma virus N gene (1).

RESULTS

Expression of the wild-type and mutant *ref(2)P* genes. As has already been described (11), the *ref(2)P* gene is divided into three exons (Fig. 1). The wild-type alleles, permissive or restrictive, code mainly for two mRNAs which differ in about 120 nucleotides at the noncoding 5′ end (indicated as E1L and E1S in Fig. 1). The mRNA heterogeneity does not affect the open reading frame. A unique 599-aa protein is predicted for all the transcripts of the permissive *p^o* allele (9). We carried out a quantitative study of transcription by dot-blotting experiments as described in Materials and Methods. Measured amounts of poly(A)$^+$ RNA purified from embryos and adult flies were applied on the membrane as a dilution series. The *ref(2)P* mRNAs were revealed by hybridization with probes of known specific activities (Fig. 2). The mRNA amounts were a function of the number of the *ref(2)P* gene copies. The *p^o/p^o* homozygous strain (Fig. 2, lane 1) accumulated twice as many transcripts as the *p^o/def* hemizygous strain (Fig. 2, lane 2). The proportions of the *ref(2)P* mRNA were calculated after quantitation. They represented at least 10^{-3} of total poly(A)$^+$ RNA

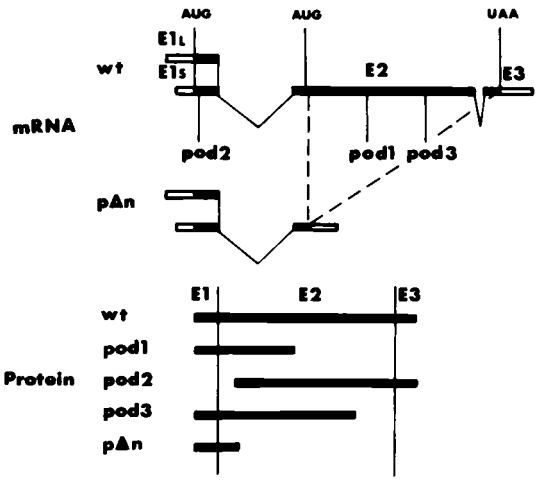

FIG. 1. Structure and expression of the *ref(2)P* gene. In the mRNA diagram, lines represent introns. In the exons, the solid blocks represent the coding region and the open blocks correspond to the untranslated ends. The variable-length 5′ end is indicated by E1L and E1S. The p^{od1}, p^{od2}, and p^{od3} mutation sites are indicated, as are the AUG codons used for translation initiation. Also shown are the mRNA of the $p^{\Delta n}$ allele and the internal *Nar*I-*Nar*I region deleted from the genomic DNA of this allele (indicated by dotted lines). The first *Nar*I site is at position 99 in the sequence of exon 2, and the second site is at position 58 in the sequence of exon 3. The proteins synthesized by the wild-type (wt) and mutant strains and by the truncated $p^{\Delta n}$ gene used in transformation are also represented.

in the wild-type male flies. Identical proportions of mRNA were present in males and females, indicating that the mRNAs accumulated in somatic tissues as well as in ovaries, which account for half of the weight of the female. Embryos contained about threefold fewer transcripts than adults (Table 1).

Three null mutations of the *ref(2)P* gene, p^{od1}, p^{od2}, and p^{od3}, were obtained from the permissive p^o allele by mutagenesis with diepoxybutane. The mutations were short deletions in the coding regions, at the beginning of exon 1 for the p^{od2} mutant and in exon 2 for the p^{od1} and p^{od3} mutants (Fig. 1). In the p^{od2} allele, the mutation generated a stop codon in place of the eighth codon following the initiation site of translation (13). The full-length *ref(2)P* mRNA was revealed in each mutant by Northern (RNA) blot analysis, as expected for such mutations (data not shown). A quantitative study of transcription was performed by dot blotting as described above. The *ref(2)P* mRNA amounts isolated from male mutants, p^{od1}/*def*, p^{od2}/*def*, and p^{od3}/*def*, were compared with those found in the wild-type p^o/*def* males (Table 1). Only the p^{od2} allele showed results similar to those of the reference strain. Although the regulatory sites of transcription are not involved in the mutations, fivefold fewer transcripts were detected in the p^{od1} and p^{od3} mutants (seen for p^{od3} in Fig. 2, lane 3). The levels of the *ref(2)P* transcripts were found to be similar in the p^o/*def* and p^o/p^{od3} strains (Fig. 2, lanes 2 and 4, respectively). As S1 nuclease mapping demonstrated, the expression of a wild-type allele was not affected by the presence of a null allele and could not stabilize the mRNAs of the p^{od3} or p^{od1} mutants in a heterozygous strain (data not shown). Thus, expression of the *ref(2)P* gene was independent of the allelic composition at the locus, and the low expression in the p^{od1} and p^{od3} mutants seems to be the result of a *cis* effect by the mutations.

TABLE 1. Proportions of the *ref(2)P* mRNA compared with levels in wild-type p^o males

Developmental stage[a]	Allele	Ratio[b]	Expt no.
Female	p^o	1	16
Male	p^{od1}	0.17	3
Male	p^{od2}	0.72	3
Male	p^{od3}	0.18	3
Male	$p^{\Delta n}$	1.11	3
Embryo	p^o	0.35	7
Embryo	p^{od2}	0.4	1
Embryo	$p^{\Delta n}$	0.30	1

[a] Embryos aged 0 to 16 h were collected.
[b] The amounts of the *ref(2)P* mRNA were measured by dot-blotting experiments as described in Materials and Methods. The results were normalized against the levels in the p^o male, accounting for the number of gene copies at the locus and the messenger size for the $p^{\Delta n}$ allele. The means were determined from independent experiments whose number is indicated in the last column.

The protein coded for by the *ref(2)P* gene has been previously identified in protein extracts of adult flies, embryos, and *Drosophila* cultured cells (21). The protein was isolated from total protein extracted from the wild-type p^o/p^o flies under denaturing conditions and was immunoprecipitated with T-REF antibodies. After separation on an SDS-polyacrylamide gel and detection by Western blotting, the Ref(2)P protein

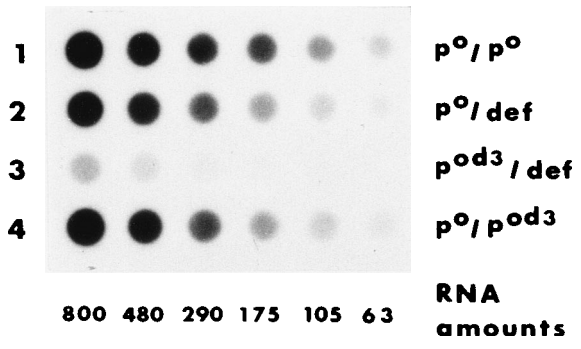

FIG. 2. Dot-blotting hybridization of *ref(2)P* mRNA. Poly(A)$^+$ RNAs were isolated from strains p^o/p^o (lane 1), p^o/*def* (lane 2), p^{od3}/*def* (lane 3), and p^o/p^{od3} (lane 4). The RNA amounts (indicated in nanograms) were dotted on the membrane. Hybridization was carried out as described in Materials and Methods and was followed by autoradiography and scintillation counting.

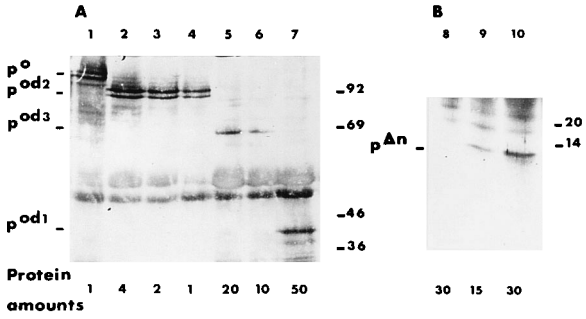

FIG. 3. Western blot analysis of the wild-type and mutant Ref(2)P proteins after immunoprecipitation. Total protein was extracted from adults of *Drosophila* strains p^o/p^o (lane 1), p^{od2}/p^{od2} (lanes 2 to 4), p^{od3}/*def* (lanes 5 and 6), p^{od1}/*def* (lane 7), p^o/p^o (lane 8), and $p^{\Delta n}/p^{\Delta n}$ p^{od3}/*def* (lanes 9 and 10). Immunoprecipitations were performed under denaturing conditions with the protein amounts indicated (in milligrams), with T-REF antibodies added in excess. Immune complexes were analyzed by SDS-polyacrylamide gel electrophoresis (gel A, 10% acrylamide and 0.26% bisacrylamide; gel B, 18% acrylamide and 0.33% bisacrylamide) and Western blotting. The blot was immunostained with T-REF antibodies as described in Materials and Methods. Molecular mass markers are indicated in kilodaltons to the right of each panel.

TABLE 2. Proportions of the Ref(2)P protein compared with levels in the wild-type p^o strain at the adult stage

Developmental stage[a]	Allele	Ratio[b]	Expt no.
Adult	p^{od1}	0.02	2
Adult	p^{od2}	0.68	4
Adult	p^{od3}	0.02	3
Adult	$p^{\Delta n}$	0.32	5
Embryo	p^o	8.25	4
Embryo	p^{od2}	0.70	3
Embryo	$p^{\Delta n}$	0.47	2

[a] Embryos aged 0 to 16 h were collected.

[b] The amounts of the Ref(2)P protein, detected by immunoprecipitation in the different strains, were compared with levels in the p^o adult flies as described in Materials and Methods, accounting for the number of gene copies at the locus and the size of each polypeptide. The means were determined from independent experiments whose number is indicated in the last column.

migrated as a protein doublet with an apparent molecular mass of 95 to 100 kDa (Fig. 3, lane 1).

The p^{od1}, p^{od2}, and p^{od3} mutants were selected for their lack of physiological functions (13); however, they synthesized the full-length *ref(2)P* mRNAs at various levels. To determine whether the null phenotype was due to a low rate of gene expression or the synthesis of a nonfunctional protein, we tested for the presence of potential translation products. The polypeptides were isolated from the protein extracts of mutant flies by immunoprecipitation under denaturing conditions and were analyzed by Western blotting as described above (Fig. 3). An additional diffuse band detected in each sample was due to antibodies used in the immunoprecipitations. In each mutant, a specific protein which differed in size and quantity from the wild-type protein shown in lane 1 of Fig. 3 was detected. The molecular masses of the proteins from the p^{od1} (Fig. 3, lane 7) and p^{od3} (Fig. 3, lanes 5 and 6) mutants were estimated at 40 and 69 kDa, respectively. They corresponded to the predicted masses of the polypeptides for which the synthesis was prematurely terminated at the mutation sites (Fig. 1). The amounts of the mutant proteins were extremely reduced. The strains were homozygous except for the p^{od1} and p^{od3} alleles. The p^{od1}/*def* and p^{od3}/*def* lines contained mutant Ref(2)P proteins at 1% of the wild-type protein level in the p^o/p^o strain and thus at about 2% for one gene copy. Alternatively, the protein from the p^{od2} mutant was at 66% of the wild-type protein level (Table 2). It was separated into two bands on the gel, as was the reference protein from the p^o allele, and its apparent molecular mass was 85 kDa (Fig. 3, lanes 2, 3, and 4). From an analysis of the *ref(2)P* gene sequence (9, 13), these results could be explained by the efficient reinitiation of translation at an ATG in the beginning of exon 2 after the mutation in exon 1. The deletion of the first 82 aa (14% of the protein sequence) corresponded exactly to the observed decrease in the molecular mass of the protein from the p^{od2} mutant. The properties of these three proteins could explain the results obtained for the transcription of the mutants (Table 1). The nonsense mutations prevented the full translation of the p^{od1} and p^{od3} mRNAs. Such messengers were unstable and were not translated efficiently (19). For the p^{od2} mutant, the translation of the 3′ end probably stabilized the transcripts, consistent with protein production at almost normal amounts.

We modified the *ref(2)P* gene in vitro to obtain the $p^{\Delta n}$ allele encoding only the N-terminal domain of the protein plus the 5′ and 3′ regulation sequences. It expressed the first 91 aa of the restrictive protein coded for by exon 1 and the beginning of exon 2 (Fig. 1). The $p^{\Delta n}$ gene was transferred into the germ line of a *Drosophila* strain. In the transformed lines, the resident alleles at the *ref(2)P* locus were replaced by crossings to establish the null p^{od3}/*def* genotype which expressed only very small amounts of a short Ref(2)P protein. Such *Drosophila* strains enabled us to study the expression and biological activity of the products from the deleted transformant gene. The accumulated amounts of the $p^{\Delta n}$ mRNA were similar to those of the wild-type strain (Table 1). A short polypeptide was detected in the $p^{\Delta n}$ line (Fig. 3, lanes 9 and 10) but was not found in the p^o strain (lane 8) or in the p^{od3}/*def* strain (data not shown). It had molecular mass of 11 kDa, comparable to the 10.3 kDa calculated from the amino acid sequence of the $p^{\Delta n}$ gene. Only threefold less protein was accumulated in the $p^{\Delta n}$ line than in the p^o line, accounting for the size of each protein (Table 2). The mRNAs were stable and normal amounts of the protein were found in adults, as expected when the coding sequence was not interrupted by a nonsense mutation in the transcripts.

Eightfold more Ref(2)P protein was found in embryos than in adults in the wild p^o strains, whereas the protein was not accumulated in the $p^{\Delta n}$ and p^{od2} strains during the early steps of development. The large amounts of Ref(2)P protein detected during embryogenesis seemed to require the synthesis of the whole protein.

Involvement of the N-terminal domain of the Ref(2)P protein in restriction. Four haplotypes of the *ref(2)P* gene have been sequenced to analyze the differences between the permissive and restrictive alleles. It has been shown that three mutations in the coding sequence of exon 1 are characteristic of the strains restrictive for sigma virus multiplication. Two codons (glutamine-asparagine) are replaced by one (glycine), and two point mutations cause amino acid substitutions (10). Exon 1 would be involved in the determination of the permissive or restrictive phenotypes. We showed above that the polypeptide coded for by this exon was precisely deleted in the protein from the p^{od2} mutant. This protein, found in large amounts, was considered to be nonfunctional during the viral cycle. However, expression of the truncated protein from the p^{od1} and p^{od3} mutants was not sufficient to investigate whether the N-terminal part of the Ref(2)P protein was involved in the control of viral replication. Thus, we constructed the $p^{\Delta n}$ gene containing mainly exon 1 from the restrictive p^p allele. The protein expressed at a high level by $p^{\Delta n}$ was complementary to that expressed by p^{od2}.

Two types of experiments were performed to study the effects of the $p^{\Delta n}$ gene product on the sigma virus. First, the efficiency of a primary infection was measured after the inoculation of virus into adult flies with various genotypes. They were injected with a dilution series of virus. The A3 sigma virus was chosen for its extreme sensitivity to the effects of the restrictive *ref(2)P* alleles. Virus multiplication was extremely weak in the restrictive *Drosophila* strains, and infection was observed only in the permissive strains under the experimental conditions used (12). This allowed us to distinguish between the different types of alleles. Sigma virus was detected in the null p^{od3}/*def* line and the permissive strains but not in the restrictive strains (Table 3). The virus did not multiply enough to be observed in the $p^{\Delta n}$ p^{od3}/*def* line, which behaved as a restrictive strain. In this line, the expression of only the first 91 aa of a restrictive protein was sufficient to determine a restrictive phenotype in the somatic tissues of an adult *D. melanogaster*.

Second, we measured the hereditary transmission of the A3 sigma virus in the presence of the $p^{\Delta n}$ gene. The virus could establish a persistent infection in the germ line cells of permissive flies and be vertically transmitted through generations. Although substantial amounts of virus were transferred into embryos from infected females, the virus had to be preserved

TABLE 3. Effects of the $p^{\Delta n}$ allele on sigma virus multiplication

Genotype of assayed flies		Detection of infection[a]	
Resident locus	Inserted gene	After injection of virus into adult flies	After hereditary transmission of virus to progeny
Null p^{od3}/def		+	+
	Permissive	+	+
	Restrictive[b]	−	−
	$p^{\Delta n}$	−	+
Restrictive p^{p}/def			−
	Permissive		+
	Restrictive[b]		−
	$p^{\Delta n}$		−

[a] More than 90% of the flies in the permissive or null contexts (+) and less than 1% in the restrictive context (−) were infected after the virus was injected into adult flies or into the adult progeny of the infected flies.

[b] The wild-type p^{p} allele and the $p^{n\text{-}i2}$ allele in which intron 2 was deleted (see Materials and Methods) were tested and showed similar results.

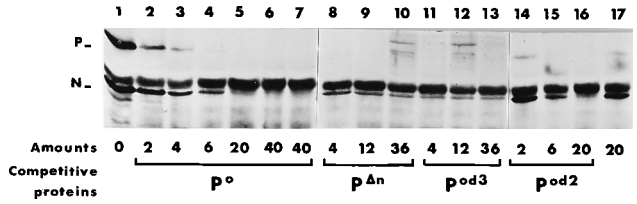

FIG. 4. Western blot analysis of the viral N protein after immunoprecipitation with anti-Ref(2)P protein antibodies under conditions of competition with the Ref(2)P protein. Total protein was extracted (at 4 mg/ml) from uninfected adults of *Drosophila* strains p^{o}/p^{o} (lanes 2 to 7), $p^{\Delta n}/p^{\Delta n}$ p^{od3}/def (lanes 8 to 10), p^{od3}/def (lanes 11 to 13), p^{od2}/p^{od2} (lanes 14 to 16), and p^{o}/p^{o} cleared of the Ref(2)P protein as described in the text (lane 17). Increasing protein amounts (indicated in milligrams) were treated with 20 μl of T-REF antibodies for 4 h at 20°C. Total protein (4 mg/ml) isolated from the p^{o}/p^{o} strain infected with the 23DA sigma virus was incubated overnight at 4°C for each sample, except for the sample in lane 7, which was used as a control. The reference sample without the competitor Ref(2)P protein is shown in lane 1. Immune complexes were analyzed by Western blotting, and viral proteins were revealed with sigma virus RNP antibodies as described in Materials and Methods.

and multiply to maintain a persistent infection during the development of all tissues. Detection of virus in the adult offspring depended on the genotypes of the offspring produced by crossings. In a first experiment, the null p^{od3}/def genotype was found at the resident locus (Table 3). Sigma virus was transmitted and multiplied in adults having the original null genotype or a permissive one obtained after transformation. But the virus was no longer detectable in the presence of restrictive alleles. Alternatively, the $p^{\Delta n}$ allele was not restrictive under these conditions and allowed the virus to grow. In another experiment, the restrictive hemizygous p^{p}/def genotype was found at the resident locus in the offspring (Table 3). This was sufficient to prevent virus multiplication. The insertion of a permissive allele by transformation restored the transmission of infection into progeny by competition between the permissive and restrictive *ref(2)P* alleles. Such a competition did not occur in the presence of the $p^{\Delta n}$ allele. We verified that these results were not due to an alteration of *ref(2)P* gene expression in the absence of intron 2. The activities of the two restrictive alleles encoding the complete Ref(2)P protein (the wild-type p^{p} allele and the $p^{n\text{-}i2}$ allele from which intron 2 was deleted in vitro) were similar in both assays (Table 3).

The $p^{\Delta n}$ allele led to a restrictive phenotype after injection of virus into an adult *D. melanogaster*. Unexpectedly, with hereditary transmission of the virus, the $p^{\Delta n}$ allele alone did not produce a decrease in infection in progeny, as would be expected for a restrictive allele (Table 3). Also, it did not compete with the p^{p} allele and was inactive in a heterozygous strain (Table 3). Thus, the $p^{\Delta n}$ gene exhibited a null phenotype during development. The transcription of this gene was not found to be modified; the only difference detected was the lack in embryos of the protein produced by $p^{\Delta n}$.

Localization of the epitopes common with those of the viral N protein in a domain of the Ref(2)P protein distinct from the restrictive site. We have previously shown (21) that the Ref(2)P protein shares antigenic determinants with the sigma virus N protein, which is associated with the viral genome. These common epitopes are scarce and are revealed by antigen-antibody cross-reactions only when the protein conformation is preserved. The two native proteins have similar structures whose roles in host-virus interactions are not yet known (21). An attractive hypothesis was that the sequence in the host protein containing the restrictive site coded also for the common epitopes. Thus, we determined whether these epitopes were found in the first 91 aa of the Ref(2)P protein by analyzing which gene product, that of $p^{\Delta n}$ or p^{od2}, possessed structures identical to those of the viral N protein. This comparison was possible because the protein expressed by $p^{\Delta n}$ was active on the sigma virus in adult flies and certainly showed a conformation at the restrictive site in exon 1 similar to that found in the restrictive protein expressed by p^{p}.

The polyclonal antibodies directed against the Ref(2)P protein were able to immunoprecipitate the N protein from extracts prepared under nondenaturing conditions from sigma virus-infected *Drosophila* strains. The viral proteins found in the immune complexes by cross-reactions were analyzed by electrophoresis and were detected on Western blots by antibodies directed against sigma virus proteins. The viral N and P proteins were precipitated from a p^{o} line persistently infected with the 23DA virus (Fig. 4, lanes 1 and 7). As was previously shown (21), the cross-reaction involved only the N protein. The P protein was also frequently precipitated under native conditions because of the formation of N-P complexes in vivo. The proportions of cross-reacting antibodies were weak under the experimental conditions used. The N protein was always in excess in the protein extracts, and the immunoprecipitated amounts were roughly proportional to the amounts of anti-Ref(2)P antiserum added (Fig. 5, lanes 11 to 13). This allowed us to undertake competition experiments with the N and

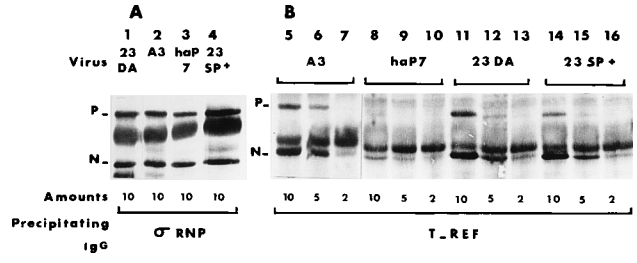

FIG. 5. Western blot analysis of the N protein coded for by different variants and mutants of the sigma virus after immunoprecipitation of the protein. The viral protein was isolated from total protein extracted from p^{o}/p^{o} flies persistently infected with the viruses as indicated. The viral protein was immunoprecipitated under native conditions as follows: directly with an excess of specific sigma virus RNP antibodies (10 μl) from 50 μg of total proteins (A) and by cross-reaction with decreasing amounts of T-REF antibodies (10 to 2 μl) from 2 mg of total protein (B). The immune complexes were analyzed by Western blotting, with sigma virus RNP antibodies being used as described in Materials and Methods. IgG, immunoglobulin G.

Ref(2)P proteins and the cross-reacting antibodies in two steps as follows. First, antibodies reacting with the N protein were depleted from the anti-Ref(2)P antiserum. Identical quantities of T-REF antibodies were treated with increasing amounts of proteins extracted from uninfected adult *Drosophila* flies with different genotypes. The cross-reacting antibodies associated with the wild protein and the mutant Ref(2)P proteins only when the common epitopes were present. Second, the unbound antibodies were detected by the addition of N protein. The same amount of total protein extracted from 23DA sigma virus-infected p^o flies was added to each sample as a source of viral proteins. The immunoprecipitated N protein was revealed on Western blots as described above. In the immune complexes, the proportions of the N protein decreased in the presence of increased quantities of the permissive protein expressed by p^o (Fig. 4, lanes 2 to 6) compared with those of the control, which was free of competitive protein (lane 1). Identical results were obtained with the restrictive protein expressed by p^p (data not shown). As a control for specificity, an extract of p^o flies was cleared of the Ref(2)P protein with an excess of T-REF antibodies. After the removal of the antigen-antibody complexes and free antibodies by the addition of protein A-Sepharose, the supernatant was used as competitor (Fig. 4, lane 17). Under these conditions, the N protein was immunoprecipitated and competition did not occur in the absence of the Ref(2)P protein.

Similarly, competition was not expected in the presence of extracts of p^{od3} and p^{od1} flies because of the almost total absence of the Ref(2)P protein (Table 2). Proteins extracted from the p^{od3}/*def* line (Fig. 4, lanes 11 to 13) were used as a control for the $p^{\Delta n}/p^{\Delta n}$ p^{od3}/*def* line (lanes 8 to 10). No clear difference was observed, and the protein expressed by $p^{\Delta n}$ was not a competitor, although the concentration of the truncated protein was 32-fold higher than that in the p^{od3}/*def* line (Table 2). In contrast, the addition of the protein expressed by p^{od2} inhibited the precipitation of the viral protein (Fig. 4, lanes 14 to 16). Therefore, in the *ref(2)P* gene, the sequence coding for the epitopes common with those of the viral N protein was found in exons 2 and 3 and was distinct from the domain in exon 1 establishing restrictivity.

Effects of a mutation in the viral N protein. To determine whether mutations could lead to the loss of the common epitopes, we studied the cross-reactivity between the anti-Ref(2)P protein antibodies and the N protein in several variants and mutants of the sigma virus. The viral strains used, A3, 23DA, *haP7*, and 23SP$^+$, differed in their abilities to multiply in the presence of the restrictive p^p allele. The A3 and 23DA strains are natural strains, with the A3 strain being much more sensitive to restrictive effects than the 23DA virus. The *haP7* mutant was obtained by mutagenesis with the 5′ fluorouracyl from the 23DA virus and was selected as being partially adapted to the effects of the p^p allele. The 23SP$^+$ strain is another variant of the 23DA virus and is likely a polymutant which is totally resistant to the effects of the p^p allele (5).

The viruses were injected into permissive p^o flies to establish a persistent infection without restriction. As a control, the viral proteins were immunoprecipitated from extracts of the infected flies by an excess of antibodies directed against the sigma virus proteins. The accumulated amounts of the N protein were identical in all cases (Fig. 5, lanes 1 to 4). The multiplication rates of these different viruses were similar in the permissive line, and the presence of the common epitopes could be quantitatively compared. Equivalent samples of the infected fly extracts were treated with decreasing amounts of anti-Ref(2)P protein antibodies. The immunoprecipitated viral proteins were analyzed by Western blotting (Fig. 5). A smaller quantity of N protein was precipitated from the *haP7* mutant (Fig. 5, lanes 8 to 10) than from the A3, 23DA, and 23SP$^+$ viruses (lanes 5 to 7, 11 to 13, and 14 to 16, respectively) when the viral proteins were in excess. The conformational structures of the N protein could be modified in the *haP7* mutant, leading to a less efficient cross-reaction in immunoprecipitation. We compared the sequences of the N genes in the 23DA and *haP7* viruses. Two differences between both of these sequences and a first published sequence of this gene were observed (1). In the first variation, the insertion of a nucleotide (A) after position 316 and the deletion of another (G) at position 348 changed the reading frame, resulting in 11 different aa in the deduced sequence of the protein. In the second variation, a synonymous point mutation (C→T) was located at position 1062. More interestingly, only one point mutation, G→A at position 1012, distinguished the *haP7* mutant from the 23DA virus in the N gene. This mutation was nonsynonymous. Arginine 404 was replaced by a lysine, which is also a positively charged amino acid.

TABLE 4. Size of sigma virus inoculum necessary to infect p^p and $p^{\Delta n}$ adult flies

Virus	Infecting units[a]	
	p^p	$p^{\Delta n}$
A3	6,750–17,000	3,240–17,000
23DA	25–60	220–1,380
hap7	1.7–7	100–620
23SP$^+$	0.7–1.4	15–30

[a] For each virus, adult flies with one functional p^p or $p^{\Delta n}$ allele were inoculated by a dilution series of the same viral suspension. The concentration of the efficient inoculum was determined by the end point dilution method (6). The number of infecting units in the inocula was measured by the same method in the permissive p^o/p^o flies. The values represent the limits of the confidence intervals at the 5% level of significance.

Comparison of restriction by the p^p and $p^{\Delta n}$ alleles. We demonstrated that the identical structures of the N and Ref(2)P proteins were not located in the protein expressed by $p^{\Delta n}$. We studied whether this property could modify restriction in the $p^{\Delta n}$ strains. The efficiencies of primary infections were compared in the p^p and $p^{\Delta n}$ *Drosophila* lines after the A3, 23DA, *hap7*, and 23SP$^+$ viruses were injected into them as described above. The concentrations of the viral suspensions were determined in the p^o/p^o flies, which were completely permissive for the viruses used. Then, the number of infecting units required to infect the restrictive flies was measured (Table 4). The increasing sensitivity of the virus to an allele paralleled the increasing amounts of the virus necessary to infect the flies. The effects of the natural p^p allele were different according to the virus studied. The more sensitive A3 virus did not distinguish between the p^p and $p^{\Delta n}$ alleles, whereas the three other viruses were clearly more restricted in the $p^{\Delta n}$ line. The greatest difference between the two genotypes was found for the *hap7* mutant, which was originally selected from the 23DA virus to be adapted to the p^p allele. However, it is not different from its parental virus in the $p^{\Delta n}$ genotype. These results seemed to indicate that exons 2 and 3, which were deleted in the protein expressed by $p^{\Delta n}$, could modulate the restrictive effects. Interestingly, the efficiency of the restriction was specifically altered with the *hap7* virus in the p^p genotype. This virus was mutated in the N protein and modified in the epitopes common with those of the Ref(2)P protein.

DISCUSSION

Particular allelic forms of the *ref(2)P* gene are able to limit the intracellular replication of sigma virus. A comparison of the sequences of two permissive and two restrictive alleles reveals 10 mutations affecting the protein sequence. Among these mutations, three located in exon 1 are characteristic of the restrictive alleles. One divergence consists of two codons (glutamine-asparagine) replaced by one (glycine), and two point mutations involve amino acid substitutions (10). Transformation experiments with the *ref(2)P* genes modified in this sequence confirmed these results (12a). We demonstrated in vivo that exon 1 was involved in the determination of the restrictive and permissive phenotypes. We analyzed the expression of null mutants of the *ref(2)P* gene and of a truncated gene constructed in vitro and inserted into the *Drosophila* germ line by transformation. The null p^{od1}, p^{od2}, and p^{od3} mutants were selected by mutagenesis from the permissive p^{o} allele. They lost their functions in the host (males are sterile) and in the control of the virus cycle. They no longer have the characteristic property of the original permissive p^{o} allele, which is to compete with the restrictive p^{p} allele. In particular, they do not counteract the inhibition of viral multiplication after injection or hereditary transmission of sigma virus in the heterozygous p^{od}/p^{p} flies. The three mutations are all located in the coding sequence (13). Nonetheless, we showed that two types of mutants had been selected. The p^{od1} and p^{od3} alleles were affected in exon 2. They accumulated few mRNAs, which were untranslated into scarce truncated polypeptides. In both cases, the sizes of the mutant proteins corresponded to the translation of the 5′ end of the messengers up to the mutation sites. The two mutants were new examples of the general phenomenon in which nonsense mutations interfere with the stability of the corresponding mRNAs. The 3′ end of the messengers was not found associated with ribosomes. The mRNAs were unstable and did not accumulate in large amounts for their translation into aberrant proteins (19). The loss of function in the p^{od1} and p^{od3} mutants could be due to the deletion of the C-terminal domain of the protein and also to a lack of *ref(2)P* gene products. Indeed, the very small detectable amounts of the Ref(2)P protein should have been sufficient to produce a null phenotype because of the dosage effect on virus multiplication described for the activity of the restrictive *ref(2)P* alleles (16). In contrast, the mRNA and the protein were found in normal amounts in the p^{od2} allele mutated in exon 1. Nonetheless, the protein synthesized was slightly shortened compared with the wild-type protein. The decrease observed in the molecular mass enabled us to determine that translation was reinitiated after the mutation on an ATG at the beginning of exon 2. After the initiation codon, it was the first in-frame ATG with a sequence close to the consensus sequence for translation initiation in *Drosophila* strains (9). In this case, the translation of the coding sequence up to the stop signal in the 3′ end stabilized the messenger. Protein synthesis remained very efficient. The protein expressed by p^{od2}, which was produced in large amounts, could be considered nonfunctional. This result suggested that the first 82 aa are necessary for protein activity in both the physiology of the host and the control of the virus cycle.

We constructed a modified *ref(2)P* gene ($p^{\Delta n}$) in vitro which expressed the complementary peptide of the protein expressed by p^{od2}. This allele was obtained by internal deletion of the restrictive p^{p} gene. It coded for the entire exon 1 and the first 34 aa of exon 2. Unlike the case with the p^{od1} and p^{od3} mutants, the gap between the termination site of translation and the 3′ end of the transcripts was roughly identical in the p^{p} and $p^{\Delta n}$ messengers. The modified gene had normal transcription and translation rates in adult flies. The protein expressed by $p^{\Delta n}$ exhibited the same biological properties as the entire restrictive protein expressed by p^{p} after injection of the A3 virus into the transformed *Drosophila* line in which only the transformant gene was expressed. The translation of exon 1 was sufficient to control virus multiplication. It produced a restrictive phenotype in the somatic tissues under the conditions of artificial inoculation. In particular, the short polypeptide synthesized seemed to be in a conformation suitable for acting on the virus. In contrast, the $p^{\Delta n}$ allele behaved as a null mutation during the hereditary transmission of sigma virus to the progeny of infected flies. We found that the amounts of the Ref(2)P protein were regulated during development in wild-type *Drosophila* flies, with eightfold more protein being detected in embryos than in adults. This accumulation seemed to be a property of the whole Ref(2)P protein. It did not occur during embryogenesis with the deleted proteins expressed by $p^{\Delta n}$ and p^{od2}. The protein expressed by $p^{\Delta n}$ was detected in small amounts at this stage and was not active against the virus during development, even though it contained the restrictive site. These results seem to indicate that a restrictive allele was efficient only when the Ref(2)P protein accumulated in embryos. They reveal a dosage effect that was already observed in the restriction levels of the homozygous p^{p}/p^{p} and hemizygous p^{p}/def strains and in the competition between the permissive and restrictive alleles (16).

These data showed that the first 91 aa of the protein expressed by $p^{\Delta n}$ contained a site for host-virus interactions. Indeed, interactions at the molecular level between the Ref(2)P and the viral P proteins have been already shown. These proteins have been found associated in complexes in the restrictive strains. Further experiments will be necessary to establish the involvement of the restriction site in the interactions with the viral P protein. Antibodies directed against the permissive Ref(2)P protein revealed homologous structures for the host protein and the viral N protein. The competition experiments with the N protein and the truncated proteins expressed by p^{od2} and $p^{\Delta n}$ and the cross-reacting antibodies showed that the common epitopes were encoded by exons 2 and 3 and were not found in the restriction site of the Ref(2)P protein. However, the epitopes were partially modified in the N protein of the *haP7* mutant, which was selected because it is less sensitive to the effects of the restrictive *ref(2)P* alleles (5). This mutant N gene differed by only one nonsynonymous point mutation from the reference N gene sequenced in the 23DA virus. This mutation determined a change of an amino acid (arginine→lysine) located in the C-terminal domain of the protein (position 404 of 450).

In rhabdoviruses, the genomic RNA and the N protein form the RNP. It is associated with viral P and L proteins acting as an RNA polymerase for transcription and replication. During a de novo infection, the N and P proteins are synthesized in large amounts after the primary transcription. They are soluble in the cytoplasm, essentially as N-P protein complexes. These proteins, if in excess, lead the viral RNA synthesis to switch from transcription to replication (20). In persistent infection, an equilibrium between the RNPs and the soluble proteins is maintained. The effects of the *ref(2)P* gene have been shown not to be equally efficient at all times of the virus cycle. Most of the restrictive effects are detected early, before viral replication. They become weaker after the increase in the viral syntheses and in persistent infection (11). These data enabled us to suggest that the Ref(2)P protein could interact with the viral RNPs, particularly via the N and P proteins. The restrictive form would be able to block or slow virus transcription and

replication at the beginning of the virus cycle. Later, the effects of the *ref(2)P* gene would be smaller because of the increase in viral syntheses during residual replications. The soluble N-P protein complexes could then compete with the RNPs for the interactions with the Ref(2)P protein to inhibit its activity.

The particular structures revealed by the common epitopes in the Ref(2)P protein would not be involved in the control of virus multiplication in a restrictive context. Alternatively, they could be used in defense mechanisms generated by the sigma virus against the effects of the *ref(2)P* gene by trapping the cellular protein in structures similar to those of the viral N-P protein complexes. This is suggested when (i) the protein expressed by $p^{\Delta n}$ is shown to be able to act against the sigma virus, even though the common epitopes have been deleted from this protein; (ii) the virus variants which were weakly inhibited by the natural p^{p} allele were found to be more restricted by the deleted $p^{\Delta n}$ allele; and (iii) the *hap7* mutant, adapted to the p^{p} but not to the $p^{\Delta n}$ allele, was modified in the structures of the N protein common with those of the Ref(2)P protein and missing in the protein expressed by $p^{\Delta n}$.

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