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Immunological Cross-Reactions and Interactions between the *Drosophila melanogaster ref(2)P* Protein and Sigma Rhabdovirus Proteins

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The *ref(2)P* gene is one of the *Drosophila melanogaster* genes involved in the inhibition of sigma rhabdovirus multiplication. The partial restriction of viral replication varies according to the *ref(2)P* alleles and virus strains and involves intracellular interactions between parasite and host products. We identified the protein encoded by the *ref(2)P* gene and produced polyclonal antibodies directed against the whole *ref(2)P* protein obtained from a recombinant baculovirus and against a part of the protein expressed as a fusion protein. These antibodies were used to study the interactions with sigma virus proteins by different immunoprecipitation techniques. We showed that the native *ref(2)P* protein shared conformation-dependent common epitopes with the viral structural genome-associated N protein. Furthermore, the cellular protein was found to be associated in complexes with the viral P protein required for RNA polymerase activity. The significance of these observations in the control of sigma virus multiplication by its host is discussed.

Viral infection involves multiple interactions between cellular and viral products at every step of parasite multiplication. Specific protein-protein interactions in bacteriophages have been shown to induce modifications in cell protein functions during the shift from host-directed to virus-directed metabolism. For example, several *Escherichia coli* proteins are used as subunits of the Oβ replicase or the T4 RNA polymerase (3, 14). Other interactions occur as defense mechanisms are mobilized against viral infection. The interferon-inducible Mx proteins, responsible for resistance especially toward negative-strand viruses in mammal cells, have been intensively studied. A model involving protein-protein interactions has been suggested, although the mechanisms which lead to the antiviral activity of these proteins are not fully understood (28).

The multiplication of the sigma rhabdovirus in *Drosophila melanogaster* is another example of a viral activity restriction. The sigma virus infects *Drosophila* species and is propagated in natural populations only by hereditary transmission. Infection can persevere in the cell cytoplasm of different host tissues, even in the germ lines, because the sigma virus is not cytopathogenic (5). Control of virus multiplication enables both infection and host survival at each developmental step of the insect. This is a natural model of selection of intracellular interactions, preserved throughout the viral cycles, leading to an equilibrium between host and virus.

Genetic analysis has led to the identification of five loci which are involved in resistance to infection. These genes, called *ref*, are polymorphic in natural *Drosophila* populations and have been defined as permissive or restrictive by comparison of their effects on sigma virus multiplication. A restrictive allele interferes with the multiplication of certain virus strains and, in particular, reduces the efficiency with which infection can be experimentally initiated. Remarkably, none of these alleles has any visible effect on the phenotype of the flies; viral infection has enabled us to identify cellular genes whose functions are not yet known in the uninfected host (19).

The *ref(2)P* gene is the most fully studied of the *ref* genes, and permissive and restrictive alleles have been characterized in both natural and laboratory strains (17). Null alleles which have lost their functions against the virus have been obtained through mutagenesis (10, 20). These alleles are fully permissive for sigma virus multiplication, although the mutations are equivalent to deletions in the coding region. This shows that the product of a permissive allele is not necessary for an efficient virus cycle but that the *ref(2)P* gene is involved in resistance to infection only in its restrictive form. Several characteristics strongly suggest that direct interactions occur between products of sigma virus genes and those of natural *ref(2)P* alleles (restrictive and permissive). (i) The *ref(2)P* gene acts only on sigma virus and not on any other viruses tested (4). This strict interdependence might implicate the recognition of specific viral products. (ii) Permissive and restrictive alleles are codominant. Their products are in competition for interactions with the virus, while the $ref(2)P^{null}$ mutants cannot compete with the restrictive alleles (26). (iii) A dose effect on virus replication is visible in homozygous and hemizygous restrictive *ref(2)P* flies (26). (iv) Sensitivity to the restrictive alleles varies widely according to the virus strains studied (19). (v) The selection of viral mutants shows that a single mutation is sufficient to escape the restrictive gene effect. Many of these adapted mutants are also found to be temperature sensitive; most temperature-sensitive mutations affect the first steps of the viral cycle (7, 8). This indicates that viral proteins are involved in the interactions with the *ref(2)P* gene products and that they are also required for the early stages of virus multiplication.

The restrictive effects of the *ref(2)P* gene are essentially detected at the beginning of the viral cycle, before replication and the increase in protein synthesis. Later effects can be observed only with the most sensitive virus strains, in particular during persistent infection (26). At the organism level, the *ref(2)P* gene participates in maintaining infection

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from one generation to another. Viral mutants selected for resistance to restrictive-allele activity are found to be more productive but also more aggressive and deleterious for the host. Fertility is affected, and there is a less efficient virus hereditary transmission (9).

These data led us to undertake the analysis of host-virus interactions at the molecular level. The *ref(2)P* gene has been cloned (10) and sequenced (13). It contains two introns and codes for mRNAs of different size as a result of the use of several promoters. These mRNAs code for a unique protein whose predicted sequence has no homology with any known protein. The sigma virus genome has been partially sequenced, and five genes have been found, as usual for a rhabdovirus. They correspond to the N nucleoprotein, the polymerase-associated P protein (previously called NS), the matrix M protein, the G glycoprotein, and the L polymerase. A sixth open reading frame could code for an additional X gene, whose expression has not been yet demonstrated (33).

In this report we identify the protein encoded by the *ref(2)P* gene and show that it shares common epitopes with the viral N protein and can be associated with at least the viral P protein in multiprotein complexes.

MATERIALS AND METHODS

***Drosophila* and virus strains.** The wild-type alleles, permissive *ref(2)P*^{o} and restrictive *ref(2)P*^{p} and *ref(2)P*^{rl}, were found in OM, Paris, and Nagore *Drosophila* strains, respectively. The *ref(2)P*^{null} alleles were obtained from the *ref(2)P*^{o} allele following mutagenesis by the PM disgenesis system for the *ref(2)P*^{hd3} mutant (10) and with diepoxybutane for the *ref(2)P*^{od1}, *ref(2)P*^{od2}, and *ref(2)P*^{od3} mutants (20). In all strains the first and third chromosomes were from the OM strain.

The 23DA sigma virus is sensitive to the restrictive *ref(2)P* alleles (8). Female OM flies which regularly transmit this virus were obtained as previously described (6). They were used to obtain, by crossing, infected strains carrying the *ref(2)P* alleles described above. Hereditary transmission was efficient in the *ref(2)P*^{p} and *ref(2)P*^{rl} flies, although the 23DA sigma virus was 100-fold less virulent in the restrictive strains than in the permissive one (8).

Production of antisera. (i) Anti-*ref(2)P* antibodies. Antigens of two origins were used. To obtain F-REF antibodies, a fusion protein was produced in *E. coli* by using the pEX bacterial expression vectors as described by Stanley (32). A *SalI-PstI ref(2)P* DNA fragment (441 bp, amino acids 113 to 259 of the predicted 599-amino-acid protein) was inserted at the carboxy terminal of the Cro-β-galactosidase gene. Colony hybridization and general DNA manipulations were carried out by the methods of Sambrook et al. (30). To produce T-REF antibodies, two recombinant baculoviruses that express the alleles *ref(2)P*^{o} and *ref(2)P*^{rl} were supplied by N. Boukhatem. cDNA inserts were isolated from pNB40 vectors and subcloned into the pACYM1 transfer vector. Recombinant viruses were obtained after transfection of *Spodoptera frugiperda* cells in the presence of infectious baculovirus DNA (25). The resultant proteins were purified on preparative gels (22). They were used to immunize rabbits (100 to 200 μg of antigen per injection) or mice (50 μg per injection) by standard procedures, with the induction of an ascitic fluid secretion in mice to raise serum production (21). Proteases were removed from sera by chromatography on CM Affi-Gel Blue resin (Bio-Rad) as specified by the manufacturer. Specific antibodies were purified on affinity columns prepared by coupling the recombinant proteins to CNBr-activated Sepharose CL-4B as specified by the manufacturer (Pharmacia). For the fusion protein, antibodies against β-galactosidase were removed by chromatography on a β-galactosidase resin.

(ii) Antibodies against the proteins of the sigma virus ribonucleoprotein. Virus was multiplied in *Drosophila* culture cells, and viral ribonucleoproteins (RNPs) were purified as previously described (34) and used to immunize mice (100 μg of total proteins per injection) as described above. Total serum was used without specific antibody purification. Sera against the viral N, P, X, or M proteins were gifts from S. Dezelee and were obtained by cloning of coding sequences in the pEX vectors to use the produced fusion proteins as antigens.

Immunoprecipitation of denatured proteins and immunoblotting. Adult flies were harvested to estimate the volume, frozen in liquid nitrogen, and ground in the presence of a mixture of protease inhibitors (CLAPA: 1 μg each of chymostatin, leupeptin, antipain, and pepstatin per ml and 8 μg of aprotinin per ml). Homogenates were diluted 1:1 with 2× SB (1× SB is 62 mM Tris-HCl [pH 6.8], 1% sodium dodecyl sulfate (SDS), and 1.25% β-mercaptoethanol), boiled for 15 min, and centrifuged twice at 12,000 × *g* for 30 min each to obtain 10 to 20 mg of protein per ml of supernatant. For immunoprecipitation, supernatants were diluted 10-fold with 10 mM Tris-HCl (pH 7.4)–150 mM NaCl–1% Triton X-100–1% sodium deoxycholate, and incubated overnight with 2 to 10 μl of purified antibodies per ml at 4°C. Immune complexes were isolated by using protein A-Sepharose beads (21).

Drosophila culture cells (cell line 77OM3) were grown and labeled with [^{35}S]methionine (100 μCi/ml; Amersham) (36). The cytoplasm and nuclei were separated as previously described (12). Proteins were denatured and immune complexes were isolated from each fraction as described above.

Immunoprecipitated proteins were analyzed by Western immunoblotting (35). Labeled proteins were revealed by membrane autoradiography, and unlabeled proteins were detected by immunostaining. Signals were amplified by the streptavidin system and revealed by enzymatic activity detection (alkaline phosphatase activity with alkaline phosphatase substrate kit I [Vestastain] and horseradish peroxydase activity with the light-based ECL system [Amersham]) (21). As far as possible in a given experiment, specific antibodies used to detect a given protein in immunoprecipitation and on the blot did not originate from the same animal species; this was done to reduce the background on the membrane as a result of immunoglobulin G.

Preparation of embryo extracts and labeling. All preparation and labeling steps were carried out at 4°C. Embryos aged 0 to 16 h were washed in Shield culture medium (36) and gently sedimented to estimate the volume. They were homogenized in a glass grinder and resuspended at 0.5 ml of embryo pellet per 2 ml of Shield medium supplemented with 5% fetal calf serum and the CLAPA antiprotease mix. The homogenates were filtered through a nylon cloth (mesh, 100 μm) and centrifuged at 600 × *g* for 5 min. For labeling, the pellets were resuspended in 0.2 ml (per 0.5 ml of original embryo volume) of methionine-free Shield medium plus 500 μCi of [^{35}S]methionine per ml and slowly stirred for times indicated in the text. Labeled extracts were pelleted at 600 × *g* for 5 min and washed in phosphate-buffered saline (PBS).

Immunoprecipitation of native proteins. (i) Method 1. Embryo extract pellets were suspended in 10 mM Tris-HCl (pH 7.4)–10 mM KCl–0.15 mM $MgCl_2$–CLAPA (2 ml per 0.5 ml of original embryo volume) for 30 min at 4°C, homogenized

with 30 strokes of a Dounce homogenizer, and centrifuged at 12,000 × *g* for 20 min. 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 overnight at 4°C. Immune complexes were precipitated by adding 10 μl of protein A-Sepharose per ml, washed five times in the immunoprecipitation buffer, and denatured in 1× SB before being subjected to immunoblotting analysis.

(ii) Method 2. Embryo extract pellets were suspended in 20 mM Tris-HCl (pH 7.4)–150 mM NaCl–5 mM $MgCl_2$–0.5% Nonidet P-40–CLAPA and treated as in method 1 but without modification of saline conditions.

Purification of the *ref(2)P* protein expressed in a recombinant baculovirus. *S. frugiperda* cells in suspension were infected with the recombinant baculoviruses described above for 3 days at a multiplicity of infection of 5 (25), collected, and washed in PBS. Purification was performed at 4°C. The cell pellet was weighed, suspended in 6 ml of 10 mM Tris-HCl (pH 8)–10 mM KCl–0.1 mM EDTA–CLAPA per g of cells for 15 min, and homogenized with 25 strokes of a Dounce homogenizer. Debris were eliminated by three successive centrifugations at 100 × *g* for 5 min, 200 × *g* for 10 min, and 7,000 × *g* for 60 min. The supernatant was diluted in 12 ml of 100 mM KCl–5 mM $MgCl_2$–0.1 mM EDTA–0.1% Triton X-100 per g of cells. Proteins were precipitated by addition of 1 volume of 1.8 M $(NH_4)_2SO_4$ and centrifuged at 11,000 × *g* for 15 min. The *ref(2)P* protein, more than 90% pure in the pellet, was solubilized in PBS, dialyzed against PBS–50% glycerol–CLAPA, and kept at −20°C.

RESULTS

Identification of the *ref(2)P* protein. To identify the protein encoded by the *ref(2)P* gene, we engineered a construction to express a part of the coding region as a fusion protein in *E. coli* and to obtain a specific antiserum. The purified antibodies (F-REF antibodies) were used to isolate the translation products of the *ref(2)P* gene by immunoprecipitation from protein extracts of adult flies prepared under denaturing conditions. Proteins isolated in immune complexes were analyzed by immunoblotting as described in Materials and Methods. As shown in Fig. 1A, specific antibodies precipitated a 95- to 100-kDa protein doublet from both wild-type fly strains, coding for the restrictive $ref(2)P^{p}$ allele (lane 2) or the permissive $ref(2)P^{o}$ allele (lane 3) for sigma virus multiplication. These strong signals were not present in a control immunoprecipitation performed without serum (lane 4) or in the $ref(2)P^{hd3}$ mutant (lane 5), which could not synthesize normal amounts of *ref(2)P* protein. The mutation is due to the insertion of a P element into the 3′ end of the gene. This mutant has physiological properties of a loss-of-function $ref(2)P^{null}$ allele and has been shown to produce very few *ref(2)P* mRNAs of abnormal size (10) (Some biological characteristics of the different *ref(2)P* alleles are summarized in Table 1). On the blot, the wide, weakly stained signals (about 50 kDa) were due to the antibodies precipitated in large amounts in the immune complexes. They were not detected in the control without serum (lane 4). The light bands found in every sample may be due to nonspecific reactions.

A noteworthy property of the *ref(2)P* gene is its unusual variability, with numerous differences in sequence between wild alleles. In particular, several deletions (48 bp total) were found within the coding sequence of the $ref(2)P^{p}$ allele compared with the $ref(2)P^{o}$ allele (15). These variations

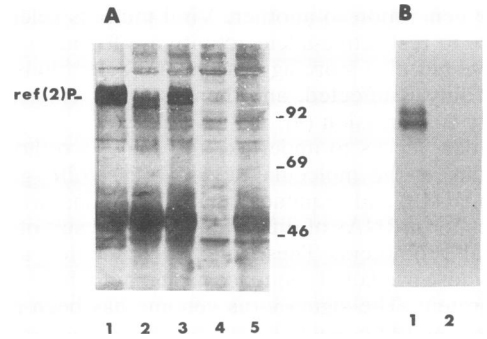

FIG. 1. (A) Western blot analysis of immunoprecipitation of the *ref(2)P* protein by F-REF antibodies from total proteins (50 mg per assay) extracted under denaturing conditions from *Drosophila* culture cells (lane 1) and from adult flies: $ref(2)P^{p}$ strain (lane 2), $ref(2)P^{o}$ strain (lane 3), $ref(2)P^{o}$ strain without serum as control (lane 4), and the $ref(2)P^{hd3}$ mutant (lane 5). The blot was immunostained with total antiserum against *ref(2)P* fusion protein by the method of detection of alkaline phosphatase activity. Molecular mass markers are indicated on the right in kilodaltons. (B) [^{35}S]methionine-labeled *Drosophila* culture cells were separated into cytoplasmic (lane 1) and nuclear (lane 2) fractions before immunoprecipitation of the *ref(2)P* protein as described for panel A and Western blotting and autoradiography as described in Materials and Methods.

account for the differences in size already noticed between the transcripts (10) and for the difference in molecular weight observed between the proteins encoded by the two alleles (lanes 2 and 3). The *ref(2)P* protein could also be detected in *Drosophila* culture cells (lane 1). It had the same apparent molecular weight as that from the permissive strain (lane 3) from which the cell line was isolated.

Purified antibodies directed against the whole *ref(2)P* protein (T-REF antibodies) were subsequently obtained after cloning a *ref(2)P* cDNA in a baculovirus and expressing the protein in *Lepidoptera* cells (see Materials and Methods). These antibodies gave identical results to those with F-REF antibodies: no additional protein was immunoprecipitated apart from the protein doublet (data not shown). The in vitro translation products of a *ref(2)P* cDNA clone were also synthesized (13). The main polypeptide, precipitated by anti-*ref(2)P* antibodies, comigrated with the heavy doublet band isolated from fly extracts. The light band was also synthesized but to a very small extent (data not shown). These results indicate that the 95- to 100-kDa protein doublet is the product of the *ref(2)P* gene, although its apparent

TABLE 1. Characteristics of *ref(2)P* alleles

Type	Allele	Interaction with sigma virus[a]	Effect on sigma virus[b]
Permissive	$ref(2)P^{o}$	+	−
Restrictive	$ref(2)P^{p}$	+	+
	$ref(2)P^{n}$	+	+
Loss-of-function mutant	$ref(2)P^{hd3}$	−	−
	$ref(2)P^{od1}$	−	−
	$ref(2)P^{od2}$	−	−
	$ref(2)P^{od3}$	−	−

[a] +, allele products that can interact with viral products and can compete for interactions; −, allele products that cannot interact or compete.

[b] +, alleles involved in resistance to infection; −, alleles with no effect.

molecular mass was significantly larger than the 76 kDa calculated from the sequence. Given the properties of the predicted *ref(2)P* protein (13), this size discrepancy could be explained by an abnormal migration of SDS-polyacrylamide gel electrophoresis. The *ref(2)P* protein is mainly hydrophilic; such proteins are known to bind smaller amounts of SDS detergent than hydrophobic species do, and the decrease in the charge/mass ratio results in a slower migration during electrophoresis. Further, the protein is rich in proline residues, a number of which are clustered with charged amino acids in different domains of the sequence. These combinations produce compact and unusual secondary structures that also result in an anomalous slower gel migration, as previously shown for the adenovirus early E1A and c-*myc* proteins (29).

Metabolic labeling in *Drosophila* culture cells revealed that the *ref(2)P* protein was partially phosphorylated in vivo and that phosphorylation alone was responsible for electrophoretic heterogeneity in the protein doublet. The protein was less abundant and accounted for only 10^{-5} to 10^{-6} of total cellular proteins (unpublished data). We also analyzed the *ref(2)P* gene expression in *Drosophila* strains persistently infected with sigma virus; there were no qualitative or quantitative differences. In particular, viral infection did not modify the proportion of *ref(2)P* protein or its phosphorylation rate. Protein localization was studied by subcellular fractionation from labeled *Drosophila* cells. Contamination between the cytoplasmic and nuclear fractions was not detectable (12). As shown in Fig. 1B, after immunoprecipitation the *ref(2)P* protein was found in the cytoplasm (lane 1), as expected for interactions with a cytoplasmic rhabdovirus. No band was observed in the nuclei (lane 2). This result was confirmed by immunocytodetection. The *ref(2)P* protein expression seems to be ubiquitous. No particular tissue distribution could be observed by immunohistochemical techniques in sections of wild-type embryos (data not shown).

Coimmunoprecipitation of the *ref(2)P* protein with two sigma virus proteins. To determine whether the *ref(2)P* protein interacts directly with proteins of sigma virus to regulate its multiplication, we used purified specific antibodies to identify by immunoprecipitation the polypeptides which could coprecipitate with the *ref(2)P* protein because of their in vivo association in protein complexes.

To compare uninfected and sigma virus-infected lines, we performed the interaction study with embryo protein extracts obtained from *Drosophila* strains expressing the different *ref(2)P* alleles. The sigma virus carrier embryos were by definition in a state of persistent infection. The 23DA sigma virus was chosen from among the virus strains sensitive to the effects of the restrictive *ref(2)P* alleles because it could be detected during persistent infection, even though its multiplication was limited in the presence of these alleles. Furthermore, the extracts of embryonic tissues could be handled like primary culture cells. The conditions of protein extraction and immunoprecipitation were adapted to avoid any denaturing treatment such as heat or ionic detergents. Cytoplasmic proteins were isolated after cell lysis at low ionic strength, and immunoprecipitations were performed with an isotonic buffer (see method 1 of immunoprecipitation of native proteins in Materials and Methods). In the experiments whose results are shown in Fig. 2A, embryo extracts were labeled with [^{35}S]methionine and then identical samples of extracted proteins were treated with various antisera to visualize and compare all the labeled proteins precipitated in the different immune complexes. Embryo proteins from

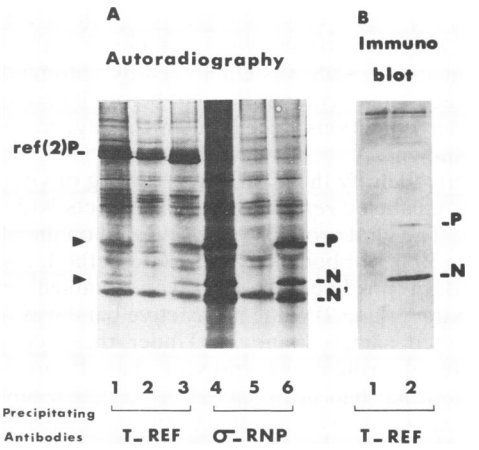

FIG. 2. Western blot analysis of immunoprecipitation of embryo extracts by various antisera. (A) Extracts were [^{35}S]methionine labeled for 5 h at 25°C. Native proteins were isolated by method 1 (see Materials and Methods). Identical samples (4×10^6 acid-insoluble cpm per ml of assay) obtained from infected (lanes 1 and 4) or uninfected (lanes 2 and 5) *ref(2)P^o* embryo and from infected *ref(2)P^p* embryo (lanes 3 and 6) were immunoprecipitated with T-REF antibodies (lanes 1 to 3) or with sigma virus RNP antibodies (lanes 4 to 6). Immunoprecipitated proteins were analyzed by Western blotting and autoradiography. (B) Uninfected (lane 1) or infected (lane 2) *ref(2)P^o* embryo extracts (1 mg of total proteins per ml of assay) were immunoprecipitated with T-REF antibodies as described for panel A. The blot was immunostained with sigma virus RNP antibodies by the ECL method.

the permissive *ref(2)P^o* strain, uninfected (lanes 2 and 5) or sigma virus infected (lanes 1 and 4), and from the infected restrictive *ref(2)P^p* strain (lanes 3 and 6) were treated with antibodies directed against the whole *ref(2)P* protein (T-REF antibodies [lanes 1 to 3]) or against the proteins of viral RNP (sigma virus RNP antibodies [lanes 4 to 6]). As expected, the use of specific antisera enabled us to isolate corresponding antigens. The *ref(2)P* protein, whose migration varied according to the expressed allele, was found in samples 1 to 3. The principal proteins of viral RNPs were visible in the infected extracts (lanes 4 and 6) but not in the uninfected control (lane 5). These proteins had the expected molecular weights and comigrated on the gel with the viral N and P proteins obtained from purified sigma virus. As indicated, the nonstructural P protein component of the RNA polymerase complex was present in large quantities in infected cells, as commonly found in other rhabdoviruses (2). The genome-associated protein could be unexpectedly separated into two forms, N and N′ (13a). The scarce N′ species was clearly detected on the overexposed autoradiograph. Many other proteins were also revealed with variable intensities. Most were present in every sample, and only a few random differences were observed between alleles. Some species were dependent on sera but not linked to infection. This background was also found after precipitation by a preimmune serum (data not shown). Nevertheless, two particular proteins coprecipitated with the *ref(2)P* protein in the presence of T-REF antibodies from infected embryos only. These proteins were visible in samples 1 and 3 (indicated by arrows) but not in uninfected sample 2. They comigrated on the gel with the viral P and N proteins. These proteins were not found in the presence of a preimmune serum or when proteins were denatured before addition of anti-*ref(2)P* anti-

bodies. In the latter case, samples of both infected and uninfected *ref(2)P*^{o} extracts were denatured with 1% SDS for 5 min at 100°C. SDS was eliminated by chromatography on an Extracti-Gel D column (Pierce) before immunoprecipitation under conditions described in the legend to Fig. 2 (data not shown).

To directly identify these proteins as being of viral origin, we treated unlabeled *ref(2)P*^{o} embryo extracts with T-REF antibodies. The viral proteins were revealed on the blot with sigma virus RNP antibodies by the ECL method. As shown in Fig. 2B, a positive response was obtained with the infected extract (lane 2) while no reactive band was found in the uninfected sample (lane 1). Under these conditions, much more N protein was present than P protein, the labeling of which had not enabled us to detect any differences. These results were confirmed by using separately the antisera raised against the N and P proteins, prepared with two fusion proteins obtained by cloning a part of the genes in pEX vectors (data not shown). This indicates that the sigma virus N and P proteins, normally involved in viral RNA synthesis, coprecipitated with the *ref(2)P* protein in the presence of polyclonal antibodies directed against the entire cellular protein. These results seemed to be specific to sigma virus. Different rhabdoviruses can multiply in *Drosophila* cells without showing any interaction with the *ref(2)P* gene. In particular, the proteins of vesicular stomatitis virus (VSV) were synthesized in very large amounts after infection in cultured *Drosophila* cells (36). However none of the VSV proteins coprecipitated with the *ref(2)P* protein in the presence of T-REF antibodies under the conditions when sigma virus proteins coprecipitated (data not shown).

Evidence of common epitopes shared by the *ref(2)P* protein and the structural genome-associated N protein of sigma virus. To analyze the nature of the protein-protein or protein-antibody interactions which determined the precipitation of the sigma virus proteins in the presence of anti-*ref(2)P* antibodies, we carried out another set of immunoprecipitations under the same conditions but with unlabeled embryo extracts. The proteins present in immune complexes were detected on the blot by a consecutive immunostaining with sigma virus RNP antibodies and T-REF antibodies by using the ECL method. The final results are shown in Fig. 3. As expected, the *ref(2)P* protein and the viral N and P proteins were revealed after precipitation of an infected *ref(2)P*^{o} extract with T-REF antibodies (Fig. 3A, lane 2). The additional bands due to added immunoglobulin G were also found with a preimmune serum (Fig. 3A, lane 1). We subjected the infected extract to four successive immunoprecipitations by adding T-REF antibodies to the supernatant of the previous precipitation, after removing antigen-antibody complexes with protein A-Sepharose. The proteins isolated in each immune complex were analyzed (Fig. 3A, lanes 2 to 5). The *ref(2)P* protein, only visible in sample 2, was entirely precipitated by the first antibody treatment. Nevertheless, viral proteins were present in every sample. The P protein was no longer detected after the third precipitation (lanes 4 and 5), but the N protein was always found even though the protein extract was cleared of *ref(2)P* protein.

Similar experiments were performed on extracts of mutant embryo expressing *ref(2)P*^{null} alleles. The null mutants were selected as alleles which had lost their functions in the cycle of viral multiplication and could no longer compete with the wild-type alleles. We studied the *ref(2)P*^{od1} and *ref(2)P*^{od3} mutants, previously obtained from the *ref(2)P*^{o} strain by mutagenesis with diepoxybutane (20). The expression of

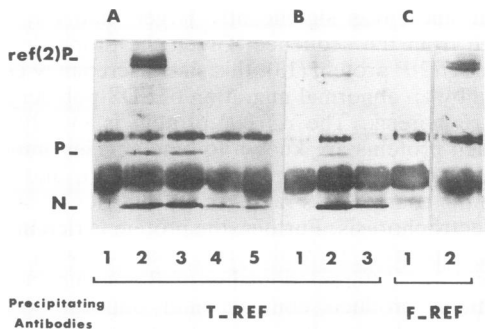

FIG. 3. Western blot analysis of immunoprecipitation of the *ref(2)P* protein isolated from embryo extracts (1 mg of protein per ml of assay) under native conditions (method 1 in Materials and Methods). The blots were immunostained with sigma virus RNP antibodies and T-REF antibodies by the ECL method. (A) A sample of infected *ref(2)P*^{o} embryo was subjected to four successive identical immunoprecipitations with T-REF antibodies (lanes 2 to 5). An identical sample was treated with a preimmune serum as a control (lane 1). (B) Extracts of uninfected (lane 1) or infected (lane 2) *ref(2)P*^{od1} mutant embryo were immunoprecipitated with T-REF antibodies. The infected sample was subjected to a second identical immunoprecipitation (lane 3). (C) Extracts of infected *ref(2)P*^{od1} mutant embryo (lane 1) and infected *ref(2)P*^{o} embryo (lane 2) were immunoprecipitated with F-REF antibodies.

these alleles was extremely limited. In both cases, truncated polypeptides were synthesized; they were shorter than the wild-type protein because the mutations are located in the coding region and were found in amounts of less than 1% of the wild-type protein (unpublished data). In these mutants, interactions with sigma virus required for control of the viral multiplication could not occur, because of the lack of effective *ref(2)P* gene products. The results of immunoprecipitations from *ref(2)P*^{od1} embryo extracts by T-REF antibodies are presented in Fig. 3B. In contrast to the uninfected sample (lane 1), the P and N proteins were precipitated from infected embryos (lane 2) even in the absence of the wild-type *ref(2)P* protein. The N protein was again revealed during a second identical immunoprecipitation of the infected extract (lane 3). Similar results were obtained with the *ref(2)P*^{od3} mutant (data not shown).

These results rule out the formation of stable complexes between the viral proteins and the wild *ref(2)P* protein isolated during immunopreciptiation, but they could indicate a cross-reaction between at least one of the viral proteins and a population of antibodies directed against the cellular protein. However, the inverse cross-reaction was not detected during immunoprecipitations with anti-sigma virus antibodies. As shown in Fig. 2A, the *ref(2)P* protein was not found either in infected samples (lanes 4 and 6) or in an uninfected extract (lane 5), even though in this latter case an immunoprecipitation could not be prevented by competition with viral proteins. *Drosophila* tissues are known to contain very low levels of the *ref(2)P* protein, and a cross-reaction could not be observed. We used the *ref(2)P* protein produced in large amounts after cloning the cDNA of the restrictive *ref(2)P*^{ts} allele in baculovirus and purified to more than 90% under its native form. Four sigma virus genes were partially cloned in pEX expression vectors to obtain antisera against the N, P, and M proteins and a hypothetical X protein, not yet characterized, which corresponds to a sixth open reading frame identified in the sigma virus genome (33). We tested the reactivity of these antisera on the baculovirus-expressed

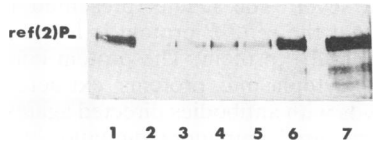

FIG. 4. Western blot analysis of immunoprecipitation of the *ref(2)P* protein expressed in baculovirus and purified (1 μg of protein per ml of assay) under conditions described in the legend to Fig. 3 with antisera raised against the sigma virus proteins N (lane 1), P (lane 2), X (lane 3), and M (lane 4) and with a preimmune serum (lane 5), sigma virus RNP antibodies (lane 6), and T-REF antibodies (lane 7). The blot was immunostained with T-REF antibodies by the ECL method.

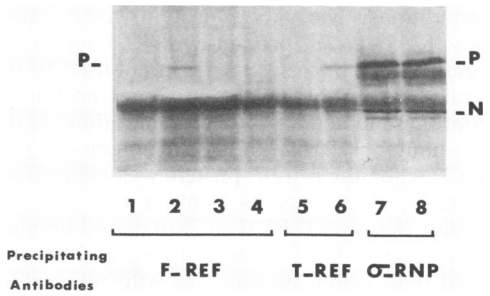

FIG. 5. Western blot analysis of immunoprecipitation of embryo proteins carried out under native conditions by method 2 (Materials and Methods). F-REF antibodies were added to samples (8 mg of protein in 4 ml) of uninfected *ref(2)P*^{rt} embryo (lane 1), infected *ref(2)P*^{rt} embryo (lane 2), and infected *ref(2)P*^{od2} mutant embryo (lane 4). The infected *ref(2)P*^{rt} extract was subjected to a second, identical immunoprecipitation (lane 3). The extracts used in lanes 5 to 8 were previously cleared of the *ref(2)P* protein by immunoprecipitation with an excess of F-REF antibodies before being subjected to the second treatment described below. T-REF antibodies were added to samples (0.8 mg of protein in 0.4 ml) of uninfected (lane 5) or infected (lane 6) *ref(2)P*^{rt} embryo. Sigma virus RNP antibodies were added to samples (0.8 mg of protein in 0.4 ml) of infected *ref(2)P*^{rt} embryo (lane 7) and infected *ref(2)P*^{od2} mutant embryo (lane 8). The blot was immunostained with sigma virus RNP antibodies by the ECL method.

ref(2)P protein under immunoprecipitation conditions as described in the legend to Fig. 3. The results are shown in Fig. 4. Equivalent amounts of protein (1 μg by assay) were treated with antibodies against the N, P, X, and M proteins (lanes 1, 2, 3, and 4, respectively) and, as controls, by a preimmune serum to evaluate the background (lane 5), by previously used sigma virus RNP antibodies (lane 6), and by T-REF antibodies (lane 7). Only the anti-N protein antiserum gave a positive signal; the others, in particular antibodies against P protein, reacted like the preimmune serum. The specific reaction detected with sigma virus RNP antibodies—directed notably against the N protein—confirmed this result. These findings strongly suggest that a population of antibodies raised against the N protein cross-reacted with the *ref(2)P* protein. This inverse cross-reaction can explain the results shown in Fig. 3; different species of anti-*ref(2)P* antibodies within the spectrum present in the polyclonal antiserum bound to the N protein, which was then immunoprecipitated. The P protein was also frequently detected, but it was not found in every experiment. In particular, it was no longer found after several successive immunoprecipitations of the embryo extract (Fig. 3A, lanes 4 and 5, and Fig. 3B, lane 3). This might be due to the existence of in vivo complexes between the N and P proteins, as already described for VSV (24), which were precipitated under our experimental conditions. These results indicate that the *ref(2)P* protein and the sigma virus N nucleoprotein share identical antigenic determinants. However, these common epitopes seem to be scarce, and the proportion of cross-reacting antibodies was weak. T-REF antibodies trapped only a part of N protein molecules during each successive precipitation (Fig. 3). The cross-reaction with anti-N protein antibodies was revealed only in the presence of large amounts of *ref(2)P* protein (Fig. 4). These structural homologies were highly dependent on protein conformation, since the antibodies recognized common antigenic determinants only in the native proteins and not in the denatured polypeptides. No cross-reactions were detected by Western blot analysis on proteins fixed on the membrane or by immunoprecipitation under denaturing and reducing conditions (data not shown).

We also investigated the cross-reactivity of F-REF antibodies purified from the partial serum obtained from the fusion protein. These antibodies did not precipitate any viral protein from an infected *ref(2)P*^{o} embryo extract when only the *ref(2)P* protein was detected (Fig. 3C, lane 2) or from an infected *ref(2)P*^{od1} mutant embryo extract (Fig. 3C, lane 1). The common epitopes might be absent from the *ref(2)P* protein domain expressed in the fusion protein, with the involved region not being encoded. However, it is also possible that conformation-dependent epitopes were not present in the truncated protein, because it would exhibit a tertiary structure quite distinct from that generated in the native protein.

Association of the *ref(2)P* protein with the sigma virus P protein. Our initial aim was to ascertain whether the *ref(2)P* protein was associated in vivo with particular viral proteins. During the previous experiments, the results obtained with F-REF antibodies which did not cross-react with the N protein showed that experimental conditions were not really suited to the isolation of potential protein complexes. However, these antibodies were also a way of testing another method of native protein extraction, to avoid misinterpretation of the results because of direct viral protein immunoprecipitation. In method 2 (Materials and Methods) the low-ionic-strength treatment and EDTA addition were eliminated. Cytoplasmic soluble proteins were isolated after membrane lysis by the nonionic Nonidet P-40 detergent in an isotonic buffer.

Results of the analysis of immunoprecipitations from different embryo extracts carried out under these conditions are shown in Fig. 5. The viral proteins present in immune complexes were detected on the blot with sigma virus RNP antibodies by using the ECL method. Another null allele was used as a control, the *ref(2)P*^{od2} mutant also obtained by mutagenesis with diepoxybutane (20). This strain was chosen because the mutant protein, which remained nonfunctional, was found in large amounts—about 50% of the wild-type protein in the adult fly—and its apparent molecular weight corresponded to 85% of the whole protein (unpublished data). Moreover, we verified that the amounts of viral proteins detected in infected embryos were very close to those found in the restrictive *ref(2)P*^{rt} allele. Equivalent quantities of the P and N proteins were immunoprecipitated with sigma virus RNP antibodies from identical protein samples of infected *ref(2)P*^{rt} embryos (lane 7) or infected *ref(2)P*^{od2} embryos (lane 8).

A significant result was obtained under these new exper-

imental conditions. The viral P protein was coimmunoprecipitated with the *ref(2)P* protein by F-REF antibodies from infected *ref(2)P*^{rt} embryos (lane 2), in contrast to the uninfected strain (lane 1). However, no viral protein was detected from the infected *ref(2)P*^{od2} mutant (lane 4) or during a second immunoprecipitation of the infected *ref(2)P*^{rt} extract (lane 3) when the *ref(2)P* protein was eliminated by the first treatment. These results confirm that there was no cross-reaction between F-REF antibodies and viral proteins. More interestingly, they showed that the P protein was precipitated only in the presence of wild-type *ref(2)P* protein. This indicates that the P protein can be associated with the *ref(2)P* protein and precipitated as a protein complex by F-REF antibodies. The N protein was not detected in sample 2, but it may have been hidden by the background, which was high as a result of the large amounts of immunoglobulin G present in the immunoprecipitates. The N protein could not be clearly detected even in a sample of infected *ref(2)P*^{rt} embryo three times higher than that shown for sample 2 in Fig. 5 (24 mg of total protein) (data not shown). However, the N protein was visible with the P protein in the control when it was precipitated after cross-reacting with T-REF antibodies (lane 6). In this case the immunoprecipitation was performed with an extract previously cleared of the *ref(2)P* protein by precipitation by F-REF antibodies, as indicated in the legend to Fig. 5. The presence of the P protein was thus not due to the formation of the protein complexes described in sample 2. We cannot conclude that the P protein was the only protein precipitated, but it was the main protein associated with the *ref(2)P* protein under these conditions.

It was interesting to compare the amounts of protein used to obtain the results presented in Fig. 5. Identical samples of *ref(2)P*^{rt} embryos (0.8 mg of protein per assay) were treated with T-REF and sigma virus RNP antibodies added in excess to trap the whole specific antigens (lanes 6 and 7, respectively). Only a rare population of anti-*ref(2)P* antibodies cross-reacted with the N protein isolated in very small proportions (lane 6) compared with the total amount present in the extract (lane 7). Ten times more protein extract was necessary to detect the interaction with the P protein (lane 2, 8 mg of protein per assay) than to visualize the common epitopes (lane 6). Thus the proportion of the P protein found to be associated with the *ref(2)P* protein was extremely reduced. The protein complexes might not be totally stable under the extraction conditions. Furthermore, the affinity between the *ref(2)P* and P proteins might be low. Nevertheless, in vivo the P protein has several confirmation states (2), and different forms may be isolated under different immunoprecipitation conditions. As proposed above, T-REF antibodies would precipitate the P protein in a complex with the N protein. Alternatively, the P protein found associated with the *ref(2)P* protein in the presence of F-REF antibodies could be a scarce catalytic form involved in transcription or replication. Indeed, the effects of the restrictive *ref(2)P*^{rt} protein are detected mainly during these steps and before the increase in protein synthesis and the formation of complexes between the de novo-synthesized N and P proteins (8).

DISCUSSION

The *ref(2)P* gene has been identified as one of the *Drosophila* genes which intervenes in sigma virus development. Particular allelic forms are able to restrict intracellular virus multiplication.

Our aim was to determine whether interactions between the *ref(2)P* and sigma virus gene products could be shown at the molecular level. The results presented in this study demonstrate that the *ref(2)P* protein is found associated in vivo with the viral P protein. The protein complexes were isolated from cytoplasmic proteins extracted from *Drosophila* embryos with antibodies directed against the cellular protein. The required immunoprecipitation conditions preserved the tertiary protein structure as well as the protein-protein interactions. Several observations argue in favor of a specific interaction. The interaction was not detected with another rhabdovirus (VSV). The P protein coimmunoprecipitated only in the presence of the wild-type *ref(2)P* protein; it was not isolated from an embryo extract previously cleared of the cellular protein. Most importantly, the viral protein was not found in a complex with the mutant protein of the *ref(2)P*^{od2} allele. This null mutant was shown to synthesize a modified protein that was slightly shorter than the wild type and was present in almost normal amounts in the adult fly (unpublished data). In this case the loss of function in sigma virus multiplication cannot be attributed to a defect in *ref(2)P* gene expression. Experiments are in progress to analyze the sequence modifications in the *ref(2)P*^{od2} protein and determine whether the deleted or mutated domains are involved in interactions with viral proteins. It is possible that the negative-binding result reflects a change in cell localization or a structural disruption in the mutant protein rather than the removal of a sequence directly necessary for protein complex formation.

The identification of protein complexes at the molecular level agrees with previously obtained physiological data which showed a direct interaction between the products of the wild-type *ref(2)P* alleles (permissive and restrictive) and those of sigma virus genes (26). Our experiments were performed with the restrictive *ref(2)P*^{rt} strain, but preliminary results indicated that the P protein was probably also associated with the protein encoded by the permissive *ref(2)P*^{o} allele. However, the small amounts of viral protein present in the infected strain made it difficult to obtain reproducible results.

Comparison of the molecular structures of the five distinct permissive or restrictive alleles of the *ref(2)P* gene revealed an unusual variability. Moreover, exon 1 contains a coding region which distinguishes the two allele types. Three close mutations, a sequence divergence and two nucleotide replacements, each modifying the amino acid sequence, are associated with the restrictive phenotype (15). A detailed analysis of this region is now being carried out by genetic transformation of *Drosophila* embryos with different modified *ref(2)P* genes to describe the changes in protein structure which determine restrictivity. The study of proteins encoded by these recombinant genes or by mutant genes such as *ref(2)P*^{od2} might show whether the domain necessary for the restrictive effect is also involved in interactions with viral proteins.

A more unexpected result was that the *ref(2)P* protein shared antigenic determinants with the viral nucleocapsid N protein. These proteins possess structural similarities strong enough to lead to antigen-antibody cross-reactions, but only when protein conformation was preserved. The common epitopes were specific to native proteins and were not due to artifactual structures found in the multiple forms of denatured polypeptides. The cross-reactivity of antiparasite antibodies with unrelated host proteins is a relatively common phenomenon, known as a molecular mimicry (27). An analysis of several hundred monoclonal antibodies directed against 11 viruses showed that 3.5% cross-reacted with host antigenic determinants in normal uninfected tissues (31).

Given the protein diversity in an organism, the probability of finding by chance a similar sequence or an identical three-dimensional configuration identified by the same antibodies in two unrelated molecules is not insignificant. Although the consequences of such similarities are not always evident, molecular mimicry has been implicated in the disruption of the host immune response which resulted in autoimmunity (27) or in parasite avoidance of host defense by failure to stimulate immune activity (11).

At the intracellular level, structural identity with a cell protein might be important for viruses if the domains concerned were involved in the formation of multiprotein complexes. In particular, they could determine new host-virus interactions. The effects might be neutral or rapidly neutralized by variant virus selection in a cell which would remain permissive. However, these interactions might also be the source of a mechanism of resistance to infection, notably if the cell protein interfered with a basic function necessary for virus growth. Such a strategy could be experimentally applied to obtain what Baltimore defined as "an intracellular immunization" (1). Friedman et al. have genetically transformed cells with a modified protein of a herpes simplex virus type 1 mutant which interferes with the function of the native protein to block transcription activation and the virus cycle (18).

By analogy with the results obtained with VSV, the P protein is known to have a key role in the principal steps of rhabdovirus multiplication, in particular in viral RNA synthesis. The P protein is associated with the L protein as a component of the RNA polymerase, and it forms complexes with both soluble and genome-associated N proteins (16). Moreover, the P protein exists in different conformational states, thought to bind to various degrees to the RNP template and to exhibit dissimilar transcription activity (2). The protein target of the *ref(2)P* protein thus appears to be a multifunctional regulation protein. Its activity might be typically modulated by new protein-protein interactions leading to a partial control of virus replication. Nevertheless, only particular P protein forms would be involved in the interactions with the *ref(2)P* protein since the P-*ref(2)P* protein complexes were isolated in small amounts. It has not yet been demonstrated that the structural identities between the *ref(2)P* and N proteins play a part in the interactions with the P protein. However, even if these similarities were due primarily to coincidence, the common epitopes have not been counter-selected during generations of sigma virus, which is known to have a highly variable RNA genome. On the other hand, most of the sigma virus mutants selected to be adapted to the restrictive *ref(2)P* alleles show modifications of proteins involved in early steps of the viral cycle as expected for the N and P proteins (7). In particular, the characteristics of the temperature-sensitive *hap*7 mutant compared with the wild-type 23DA sigma virus are very similar to those of a VSV mutant of the P protein, *ts*OII52 (23). The study of the behavior of these mutants toward the *ref(2)P* protein would allow us to test different hypotheses about the causes of the restrictivity adaptation. Interactions with the P protein could remain despite the mutations; in this case the effects of the *ref(2)P* gene would be modified to become permissive. However, the interactions could no longer be detected with certain mutants. In both cases the most likely cause would involve mutations in the P protein. However, the study of the cross-reactivity between the N protein and anti-*ref(2)P* antibodies might indicate whether the virus could escape the cellular gene control by structural modifications in the N protein, leading to the loss of the common epitopes. By coevolution the N and P proteins would establish new protein-protein interactions no longer in competition with the *ref(2)P* protein. If such mutants were selected, they would provide a strong argument in favor of common epitopes between the *ref(2)P* and N proteins connected to interactions with the P protein.

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