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Isolation of Nonsense Mutants of Lipid-Containing Bacteriophage PRD1

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We isolated nonsense mutants of bacteriophage PRD1, a lipid-containing polyhedral virus capable of infecting many genera of gram-negative bacteria. These mutants were grouped into 19 classes on the basis of genetic complementation and sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis. PRD1 infection led to the synthesis of at least 25 viral proteins, 17 of which were components of mature virions. The synthesis of proteins fell into the following three classes: very early, middle early, and late. Two of the very early proteins, P1 and P8, had an effect on DNA synthesis, host protein synthesis shutoff, and the turning on of middle and late protein synthesis. Another very early protein, P12, was involved in the shutoff of early protein synthesis. Two genes were identified as affecting lysis of the host. One appeared to be a lysin, whereas the other was an accessory lytic factor.

The members of the PRD1 phage group are very closely related, and results obtained for one member are almost always true for the other members (1, 11). The members of this bacteriophage group infect gram-negative bacteria of many genera if the bacteria carry plasmids belonging to the P, N, or W incompatibility groups (2). PRD1 is a polyhedral bacteriophage that has a DNA chromosome (15) and contains lipid, as shown for bacteriophages PR4 (17) and PR5 (22). Several studies have already been made of the composition of the virions and physiology of infection in this class of viruses (1, 3). An electron microscopic study of the development of phage PR4 has also been done (10).

We have embarked on a study of the assembly of bacteriophage PRD1. In this paper we describe the isolation of nonsense mutants of this bacteriophage and the classification of these mutants into groups on the basis of their genetic complementation behavior and their patterns of protein synthesis.

MATERIALS AND METHODS

Strains. *Salmonella typhimurium* LT2 carrying drug resistance plasmid pLM2, which has amber mutations in the *bla* and *tet* genes, was the standard nonsuppressor host for the mutant studies. This strain was designated LT2(pLM2). The suppressor strains used were strain PSA, which was derived from LT2(pLM2) by drug selection (13), and *S. typhimurium* DB7154 and DB7156, both of which carry pLM2. The latter two strains insert serine and tyrosine, respectively (21). Strain PSA has a pattern of suppression that indicates glutamine insertion. Strain MS1550(pLM2) is a UV-sensitive strain of *S. typhimurium* that was used for labeling of phage proteins synthesized in infected cells (23).

Bacteriophages. PRD1 was obtained from R. Olsen; nonsense mutants *sus51*, *sus52*, *sus53*, *sus54*, *sus55*, and *sus56* were obtained from M. Kageyama (9).

Bacteria were grown in either LC or M8 medium (19) at 37°C. Phage was prepared from confluently lysed soft agar layers by using LC medium, and our procedure yielded titers between 5×10^{10} and 1×10^{12} PFU/ml. These stocks were pelleted and suspended in M8 salts at a fourfold concentration for radioactive labeling studies.

Mutagenesis was done by growing the suppressor strain in LC medium to a titer of 3×10^8 PFU/ml, infecting with wild-type PRD1 at a multiplicity of infection of 10, and adding nitrosoguanidine to a concentration of either 25 or 40 μg/ml from 20 to 45 min postinfection. The culture was then centrifuged, suspended in LC medium, and incubated until lysis occurred. Mutagenized phage were plated with suppressor hosts, and plaques were picked with sterile toothpicks and tested on grids of lawns containing the suppressor host or LT2(pLM2). Presumptive mutants were picked, diluted, and plated onto both strains, and pure plaques were used to prepare high-titer stocks.

Radioactive labeling. MS1550(pLM2) was grown overnight in M8 medium containing proline, histidine, serine, glutamic acid, leucine, and biotin as supplements. The culture was diluted and grown to a density of 3×10^8 CFU/ml, and 20-ml portions were irradiated with 3,600 ergs/mm^2. Samples (1 ml) were infected with 10 μl of phage concentrate in tubes (25 by 150 mm) at 37°C, and 10 μCi of [^{35}S]methionine was added at different times. To stop the labeling, cells were diluted with cold LC medium, centrifuged, suspended

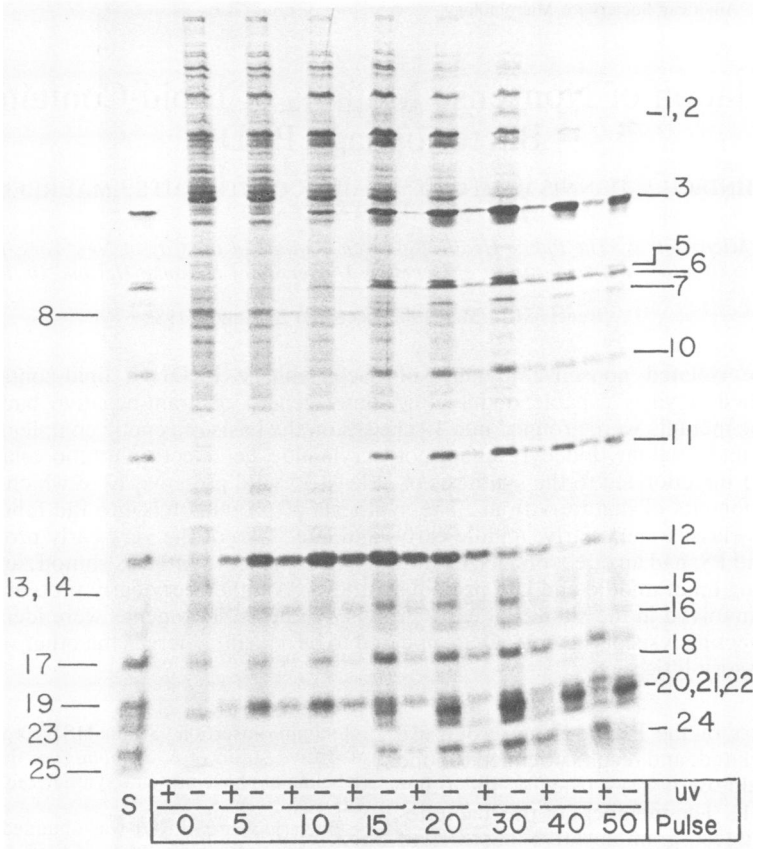

FIG. 1. Autoradiogram of extracts of UV-irradiated (+) and non-irradiated (−) MS1550(pLM2) cells infected with PRD1. Cultures were labeled for 5 min at the times indicated (in minutes). Lane S contained an irradiated infected culture labeled at 40 min.

in 0.1 ml of sample buffer (20), and boiled for 2 min.

Gel analysis. Polyacrylamide gels for proteins were prepared as described by Bamford et al. (1), except that 15.5% acrylamide was used and the pH of the running gel was 8.6. The gels were 170 mm long. Molecular weights were estimated by comparing the positions of bands with the positions of bacteriophage ϕ6 bands, which ranged in molecular weight from 82,000 to 6,000 (5). DNA samples were run on composite 2% acrylamide–0.5% agarose gels (20) and stained with ethidium bromide.

Genetic recombination and complementation. Mutants were tested against each other by the plate spot test for complementation and recombination, using LT2(pLM2) (6). Because of the high reversion frequencies observed for most of the mutants (10^{-4} to 10^{-5}), the amount of phage applied to the plates was limited, and the appearance of plaques on the test plate usually indicated recombination, as well as complementation. Plaques in excess of the plaques obtained for self tests were always found to be wild type. In addition to the spot test, which clearly delineated most groups, we also performed a quantitative complementation test involving mixed infection of LT2(pLM2) in LC broth, centrifugation, resuspension before lysis, and subsequent plating on suppressor strain PSA. Positive complementation was indicated by a yield that was 3- to 50-fold greater than the background value for a self test and a burst size of 30 to 150.

A recombination analysis of the mutants was performed by infecting cultures of strain PSA at a density of 6×10^8 CFU/ml with mutants at a multiplicity of infection of 10, incubating the cultures for 10 min at 37°C, diluting by a factor of 100, allowing lysis to occur, and finally plating on nonsuppressor strain LT2(pLM2) and suppressor strain PSA. We found recombination frequencies of 3 to 4% between most complementation groups.

Lysozyme activity. The assay of Sekiguchi and Cohen (18) as modified by Rao and Burma (16) was used with PAO1(pLM2) cells as the substrate. This assay involves the loss of turbidity in suspensions of chloroform-treated cells due to lysozyme action. Extracts of infected LT2(pLM2) or DB7156(pLM2) cells were prepared by infecting cells in LC medium, harvesting at 45 min postinfection, suspending the cells in 50 mM Tris (pH 8) at a density of 3×10^{10} cells per ml, freezing, and sonicating. In this assay, 4 μl of wild-type extract resulted in an optical density change of 0.18 U/ml per min.

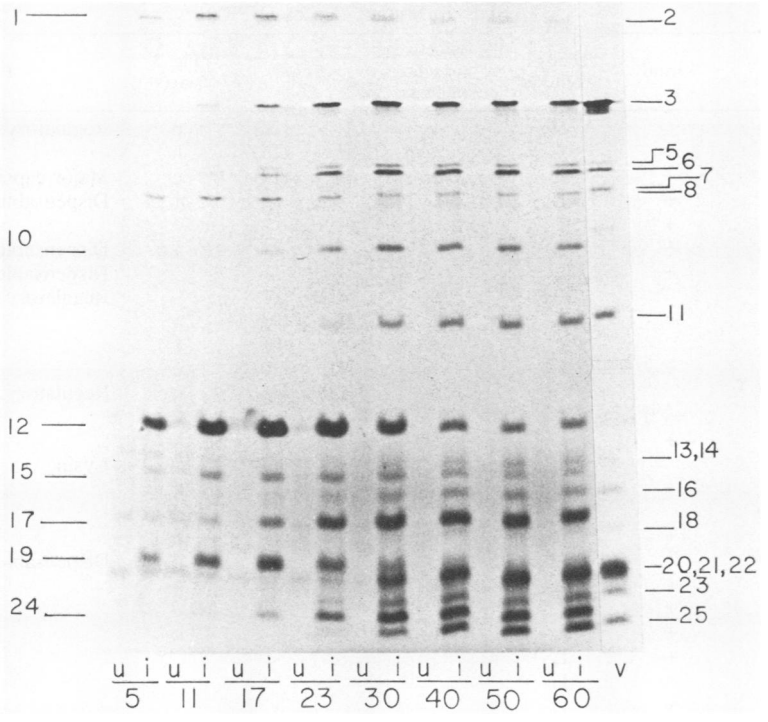

FIG. 2. Autoradiogram of extracts of MS1550(pLM2) cells that were infected with PRD1 after UV irradiation and labeled for 3 min at the times indicated (in minutes). u, Uninfected cells; i, infected cells. Lane v contained purified virions. The upper portion of the autoradiogram was exposed three times as long as the rest of the preparation to enable visualization of proteins P1 and P2.

RESULTS

Proteins specified by PRD1. PRD1 induced the synthesis of at least 25 proteins in infected cells (Fig. 1), and 17 of these proteins were found in purified phage particles (Fig. 2). The timing of the synthesis of phage proteins was followed in both UV-irradiated and untreated cells (Fig. 1 and 2). There appeared to be at least two groups of proteins on the basis of the timing of appearance in infected cells. The proteins belonging to early group (P1, P8, P12, P15, and P19) were synthesized soon after infection, whereas the later proteins started synthesis about 15 min postinfection. Lysis occurred at about 60 min postinfection. We show below that the early group could be split into very early and medium early (see Fig. 4), with P1, P8, and P12 being the very early proteins and P15 and P19 being the medium early proteins. The patterns of synthesis were not very different with irradiated and non-irradiated cells; however, in the latter case host proteins obscured some of the phage bands (Fig. 1). It was also apparent that the synthesis of some early proteins, such as P1, P8, and P19, was greatly diminished later in infection. All of the proteins found in virus particles were in the late class. Many of the proteins migrated very similarly in gels, and in many cases the identification of specific bands depended on the gel patterns obtained with nonsense or missense mutants of the phage and a careful comparison of many gel patterns. This was especially true for proteins P20, P21, and P22, which were identified primarily in purified virions (12).

Isolation of mutants. Three sets of mutant isolations were performed by using different suppressor strains. The distribution of classes obtained in each set differed. The yield of mutants was approximately 0.2%, but about one-half of the mutants exhibited reversion rates that were so high (more than 0.1% revertants in lysates prepared from single plaques) that they could not be handled easily. A total of 248 mutants were analyzed for the pattern of protein synthesis in infected cells and for genetic complementation. Almost all mutants showed some protein bands missing or altered in gels. Complementation tests resulted in the formation of 17 genetic complementation groups (Table 1). Many of the mutants could not be grouped because of leakiness. A comparison of the genetic data with the gel analysis results led to the assignment of 14 proteins to specific genetic

TABLE 1. Mutant classes of PRD1

Protein	Mol wt ($\times 10^3$)	Virion	Time of synthesis[a]	Defective nonsense mutants	Class[b]		Fig. 3 lane(s)	Function
P1	70		VE	+	I	(20)	p	Regulatory, DNA synthesis
P2	70	+	L	+	II	(15)	h	
P3	42	+	L	+	III	(14)	c	Major capsid protein
P4		+	L			—[c]	j	Dispensable
P5		+	L					
P6	35	+	L			—[c]	j,o	Dispensable
P7		+	L			—[c]		Dispensable
P8			VE	+	VIII	(2)		Regulatory, DNA synthesis
P9		+	L	+	IX	(9)	a	
P10	26		L	+	X	(8)	f	
P11	23	+	L	+	XI	(30)	e	
P12	20		VE	+	XII	(4)	n	Regulatory, DNA synthesis
P13		+(?)	L					
P14		+	L	+	XIV	(1)	k	
P15			ME	+	XV	(17)	m	Lysin
P16	11	+	L	+	XVI	(1)	q	
P17	10		L	+	XVII	(4)	d	
P18		+	L	+	XVIII	(2)	l	
P19	9		ME		—	(2)[c]	p	Dispensable
P20		+	L	+	XX	(2)	g	
P21		+	L		—	(1)[c]	o	
P22		+	L	+	XXII	(3)	k	
P23		+	L					
P24			L					
P25		+	L					
?					A	(6)		Lytic factor
?					B	(2)	j	
?					C	(1)		
?					D	(1)		

[a] VE, Very early; ME, medium early; L, late.

[b] Class refers to the genetic complementation group in all cases except class XIV, which was leaky and was identified only from the gel pattern. The numbers in parentheses denote the number of mutants assigned to each class.

[c] —, We obtained mutants that lacked these proteins or had altered migration but were able to propagate.

classes (Table 1). Genetic classes were designated with roman numerals corresponding to the gel band number of the protein product in the cases where this was known. Therefore, class XI denotes those mutants that are missing protein P11. Classes that have not yet been assigned to specific proteins were designated A, B, C, and D.

Recombination tests showed that mutants could recombine, and recombination frequencies were found to be between 4 and 8% in two-factor crosses between members of separate complementation groups. Crosses within complementation groups resulted in frequencies ranging from as high as 2% for mutants with mutations in large genes, such as classes I and III, to unmeasurably low values for mutants with mutations in smaller genes. However, there was no consistent relationship among the intergenic recombination frequencies of the various classes. We established a genetic and physical map of PRD1 on the basis of marker rescue of mutants by a set of cloned fragments of PRD1 in pBR322. The genetic classes described in this paper are consistent with that map, and this will be the subject of another report. Class XII mutants, involving P12, were very leaky and therefore could not be tested genetically. This class was defined solely on the basis of the appearance of fragments of protein P12 (Fig. 3, lane n, and Fig. 4, lanes k and l). All members of this class gave similar physiological patterns (Fig. 4, lanes k and l).

Figure 3 shows an autoradiogram illustrating the patterns obtained with various mutant classes. Table 1 lists each of the proteins identified in our gels and our results with obtaining mutants affecting the production of those proteins. The only proteins for which we did not isolate nonsense mutants are P4, P5, P13, P21, P23, P24, and P25. The latter three proteins were estimated to have molecular weights of less than 10,000. We found mutants that were missing proteins P4, P6, P7, and P19 with no apparent effect on phage propagation, although P6 mutants had a smaller plaque size. A mutant designated *sus116*

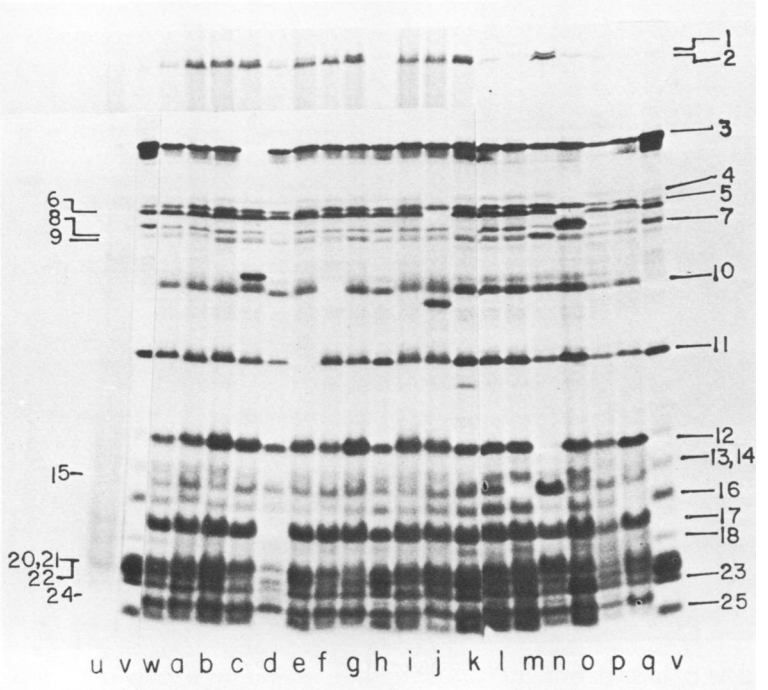

FIG. 3. Autoradiogram of extracts of MS1550(pLM2) cells infected with PRD1 *sus* mutants after UV irradiation. Cultures were labeled for 5 min at 45 min postinfection. The upper portion of the autoradiogram and lanes l to v were exposed three times as long as the rest of the preparation. Lane u, uninfected; lane w, wild type; lane a, *sus1* (class IX); lane b, *sus100* (class A); lane c, *sus24* (class III); lane d, *sus151* (class XVII); lane e, *sus44* (class XI); lane f, *sus13* (class X); lane g, *sus30* (class XX); lane h, *sus198* (class II); lane i, *sus42* (class XXII); lane j, *sus79* (class B); lane k, *sus234* (class XIV); lane l, *sus148* (class XVIII); lane m, *sus126* (class XV); lane n, *sus156* (class XII); lane o, *sus116*; lane p, *sus182* (class I); lane q, *sus167* (class XVI); lane v, virions.

had an increased mobility of protein P21 in gels (Fig. 3, lane o, and Fig. 4, lane g).

Revertants of representatives of each class of mutants were analyzed for recovered synthesis of the deficient or fragmented protein. In all cases where the band was identified on gels (Table 1), the revertants recovered synthesis of the protein. Mutant *sus234* (class XIV) was the only anomalous case. Protein P14 was missing in this mutant, but three of three revertants gained a new band of higher apparent molecular weight. P6 was missing in several classes (Fig. 3, lanes j and o). We obtained revertants that still lacked protein P6.

Assignment of roles for proteins. (i) DNA synthesis. Infected nonsuppressor cells that were not irradiated were infected with mutants to study both phage DNA synthesis and shutoff of host protein synthesis. Figure 5 shows the DNAs present in cells infected with wild-type phage and various mutants. This figure shows that normal phage caused the appearance of a band (arrow) at exactly the position found for phage DNA. This band appeared in all classes of mutants except classes I and VIII, which involve proteins P1 and P8, respectively. Mutants of class XII, involving protein P12, showed a diminution in the amount of phage DNA. The same was found for *sus239*, which either overproduces P17 or is also a mutant in P12 but whose specific lesion is not known.

(ii) Host protein synthesis shutoff. The effects of mutations on host protein synthesis shutoff are shown in Fig. 4. It is apparent that class I and class VIII (data not shown) defects were the only defects that prevented host protein synthesis shutoff (Fig. 4, lanes m and n). It is also apparent that the class XII mutants (protein P12) seemed to continue the synthesis of some very early and medium early proteins. Note that proteins P1 and P19 continued to be made late in cells infected with these mutants (Fig. 4, lanes k and l). Another interesting point is that in class I and VIII mutants we found weak synthesis of almost all proteins in UV-irradiated cells (Fig. 3, lane p) but synthesis of only P12 and P3 in non-irradiated cells (Fig. 4, lanes m and n). Although visible, the synthesis of P3 was far below the level normally found.

On the basis of the finding that the only protein synthesized at a relatively high rate by class I and VIII mutants in non-irradiated cells

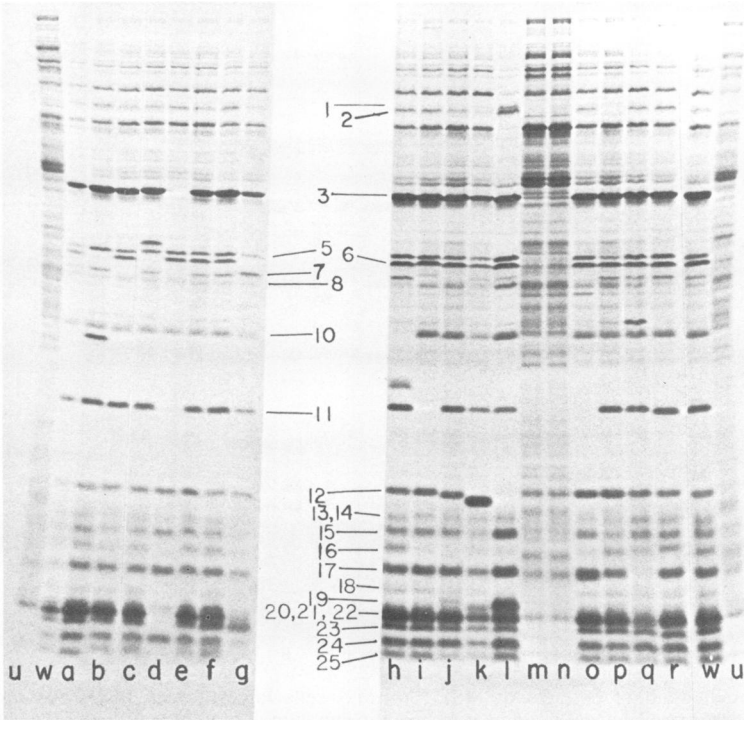

FIG. 4. Autoradiogram of extracts of MS1550(pLM2) cells infected with mutants of PRD1 with no prior UV irradiation, illustrating host protein synthesis shutoff and polarity. Labeled methionine was added at 40 min postinfection. Lane u, Uninfected; lane w, wild type; lane a, *sus79* (class B); lane b, *sus234* (class XIV); lane c, *sus117* (class A); lane d, *sus14* (class III); lane e, *sus148* (class XVIII); lane f, *sus42* (class XXII); lane g, *sus116*; lane h, *sus191* (class X); lane i, *sus186* (class XI); lane j, *sus167* (class XVI); lane k, *sus164* (class XII); lane l, *sus156* (class XII); lane m, *sus182* (class I); lane n, *sus181* (class I); lane o, *sus172* (class XI); lane p, *sus170* (class II); lane q, *sus169* (class XVII); lane r, *sus1* (class IX).

was P12, we assigned P1, P8, and P12 to the very early class. The remainder of the early proteins. P15 and P19, were not observed under these conditions (Fig. 4, lanes m and n). All of these proteins were synthesized earlier than 15 min postinfection (Fig. 1 and 2), at which time late protein synthesis began. The band for P8 was obscured in extracts of non-irradiated cells. It seemed that this protein was also present in the very early group.

(iii) Lysis. Mutants were tested for the ability to lyse infected nonsuppressor cells. Class I and VIII mutants were somewhat defective, but because of their pleiotropic effects this was expected. Two other genetic classes showed lysis defects. Class A, which was leaky and formed plaques with halos, was defective in lysis but caused lysis if chloroform was added to infected cells. The missing protein in this class has not been identified. The other class was class XV, which was missing P15. Cells infected with mutants of this class did not lyse even in the presence of chloroform.

Lysozyme activity could be assayed in extracts of infected cells, as described above. When extracts of infected cells were tested (Table 2), we found that class XV mutants did not induce lysozyme activity, whereas class A mutants did. On the basis of these findings, we conclude that class XV mutants affect the phage lysozyme, which is protein P15, and that class A defines an accessory lysis protein which is so far unidentified but which might act much as proteins found in other systems (8, 14).

DISCUSSION

PRD1 induces the synthesis of at least 25 proteins, and 17 of these are found in the virus particles. These proteins range in molecular weight from 70,000 to less than 10,000. The pattern of timing of synthesis includes the following three classes: very early, medium early, and late. The very early class includes just three proteins, P1, P8, and P12; P1 and P8 have an effect on host protein synthesis shutoff, phage DNA synthesis, and the turning on of medium early and late phage protein synthesis. The observation that mutants defective in the syn-

thesis of P1 or P8 do not synthesize medium early and late proteins in normal nonsuppressor cells but do synthesize these proteins in UV-irradiated cells may indicate that control is effected through an inhibition of *rho*-mediated transcription control. Fassler and Tessman (7) have shown that UV-irradiated cells behave as if they are deficient in *rho*. Phage lambda uses *rho*-mediated control to effect the decision to lysogenize or cause virulent infection, as well as to determine the timing of late protein synthesis (4). In the absence of protein P12 (Fig. 4, lanes k and l) the synthesis of early proteins P1, P8, and P19 continues late in infection. It appears that P12 may be involved in the shutoff of early protein synthesis. Mutants in P12 are leaky, and it seems likely that the role of this abundant early protein is regulatory. Only two of the genes that define early proteins yielded tight mutants. These are classes I and VIII (proteins P1 and P8). The remainder may play nonessential roles much as was found for T7 early proteins (20).

Two genetic groups with lysis functions have been identified. Class A proteins determine a lysis function that may be similar to gene S of lambda (8). These proteins appear to destabilize the host membrane late in infection. Gene XV determines P15, which appears to be a lysin. Of the 25 proteins identified on gels, we have located 15 in genetic groups that indicate necessary functions. At least four proteins seem to be dispensable since mutants missing these proteins are able to form plaques.

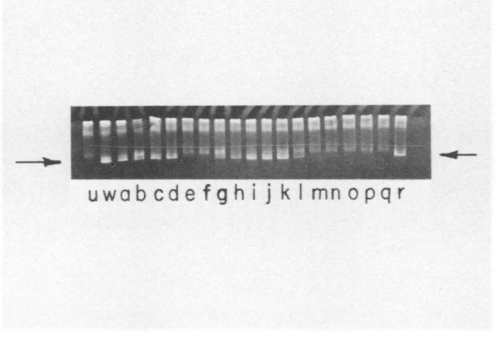

FIG. 5. Ethidium bromide-stained gel showing the DNAs in MS1550(pLM2) cells infected with mutants of PRD1. Lane u, uninfected; lane w, wild type; lane a, *sus1* (class IX); lane b, *sus169* (class XVII); lane c, *sus170* (class II); lane d, *sus172* (class XI); lane e, *sus181* (class I); lane f, *sus182* (class I); lane g, *sus156* (class XII); lane h, *sus164* (class XII); lane i, *sus167* (class XVI); lane j, *sus186* (class XI); lane k, *sus191* (class X); lane l, *sus239*; lane m, *sus196* (class I); lane n, *sus114* (class I); lane o, *sus159* (class I); lane p, *sus173* (class I); lane q, *sus233* (class VIII); lane r, *sus198* (class II).

TABLE 2. Lysin activity of cell extracts

Suppressor	Extract of cells	Infecting phage	Class	Activity[a]
−	LT2(pLM2)	Wild type		0.90
−	LT2(pLM2)	*sus56*	A	0.75
+	DB71(pLM2)	*sus56*	A	0.73
−	LT2(pLM2)	*sus126*	XV	0.001
+	DB7156(pLM2)	*sus126*	XV	0.005
−	LT2(pLM2)	*sus240*	XV	0.000
+	DB7156(pLM2)	*sus240*	XV	0.004

[a] Change in optical density at 470 nm per minute with 20 μl of extract added to 1 ml, as described in the text.

We have not found nonsense mutants for proteins P21, P23, and P25, which are the three smallest proteins of virions. These proteins constitute a major part of the membrane proteins of the virions (unpublished data), and therefore it would be very interesting to obtain mutants for them. Protein P24 is a small protein not found in virions, and we have not found nonsense mutants for this protein. We have not been able to establish a genetic map by two- or three-factor crosses of mutants. The frequencies of recombination appear to be independent of distance between markers except in the case of very close linkage (intragenic). We have established a map of the PRD1 genome by marker rescue of the nonsense mutants on strains carrying fragments of the PRD1 genome cloned into plasmid pBR322, and this will be the subject of a subsequent report.

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