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Genetic Analysis of Bacteriophage ϕ29 of *Bacillus subtilis*: Mapping of the Cistrons Coding for Structural Proteins

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Four phage ϕ29 suppressor-sensitive mutants of cistron O have been examined for production of ^{14}C-labeled viral-specific proteins in restrictive infections of *Bacillus subtilis* and fail to produce the protein of the viral neck lower collar. Cistrons O and F have been placed on the genetic map, containing 12 cistrons, by three-factor crosses. The phenotypes of five cistron J mutants have been analyzed by sodium dodecyl sulfate gel electrophoresis and autoradiography, and in three instances fragments of the normal polypeptide were detected. Three-factor crosses with these mutants and a virus with a clear plaque phenotype were used to initiate the mapping of cistron J and the determination of the orientation of transcription in this map region.

The *Bacillus subtilis* phage ϕ29 has a unique complex morphology (2), and seven viral structural proteins resolved by electrophoresis have been identified as components of the head, neck, and tail (12). The genome of ϕ29 is a linear DNA duplex with a molecular weight of 11×10^6 (2). A genetic map has been constructed by three-factor crosses with suppressor-sensitive (*sus*) mutants that defines the order of 10 of 13 cistrons (18). A map containing 17 cistrons has been constructed by two-factor crosses of temperature-sensitive and *sus* mutations (13). A maximum of 23 ^{14}C-labeled ϕ29-specific proteins has been resolved in lysates of UV-irradiated *B. subtilis* by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography (7, 9, 11, 15). Examination of the patterns of ϕ29-specific protein synthesis after infection by *sus* mutants under restrictive conditions has begun and will be discussed when appropriate (4, 5, 11).

In this communication we report the identification of a new cistron, cistron O, and its product, the lower collar protein. Cistrons F and O, coding for structural proteins or their regulation, have been positioned on the genetic map by three-factor crosses, and we have mapped mutants in cistron J. The product of this cistron is cleaved to generate the neck appendage of the virion (4, 6, 9).

MATERIALS AND METHODS

Phage and bacteria. Phage ϕ29 and a clear plaque mutant of spontaneous origin, ϕ29c, were employed in this study (4). *sus* mutants of ϕ29 or ϕ29c were isolated after mutagenesis with hydroxylamine (18) or bromodeoxyuridine (4). The cistron O mutants and mutant J874 were isolated by the bromodeoyxuridine method (4). All mutant viruses used to obtain the profiles presented in Fig. 1 contained a mutation with the phenotype of delayed lysis (13).

The properties of the permissive host *B. subtilis* L15 and the nonpermissive host *B. subtilis* SpoA12 have been described (18).

Lysate production and bacteriophage assay. Our methods and media for lysate preparation have been described (3, 17), and standard phage techniques were used (1). The phage for genetic experiments were stored in Difco antibiotic medium 3. Viruses and lysates to be examined by electrophoresis and autoradiography were prepared in the minimal medium M40 (16) supplemented with tryptophan (50 μg/ml), and input phage for these experiments were in TMS buffer (0.05 M Tris-hydrochloride, pH 7.8, 0.1 M NaCl, 0.01 M $MgCl_2$).

Genetic methods. Qualitative and quantitative complementation, two-factor and three-factor crosses, and recombinant virus construction have been described (4, 18).

To position the clear (c) mutation a standard two-factor cross was employed (18) and the recombination frequency was determined. Then 204 plaques, chosen at random, were transferred from the permissive indicator *B. subtilis* L15 by toothpick to plates seeded with the permissive indicator and incubated at 37 C overnight. This gave a more reliable indication of the proportion of the clear parent virus in the lysate than simple observation of plaques. The procedure was repeated with a random sample of plaques from the nonpermissive indicator and the percentage of recombinants with the clear phenotype was determined.

Our standard bacteriophage assay does not permit us to distinguish the clear plaque after ϕ29c infection from the plaques formed on *B. subtilis* L15 by any *sus* mutant of cistron J. Four hundred and eight wild-

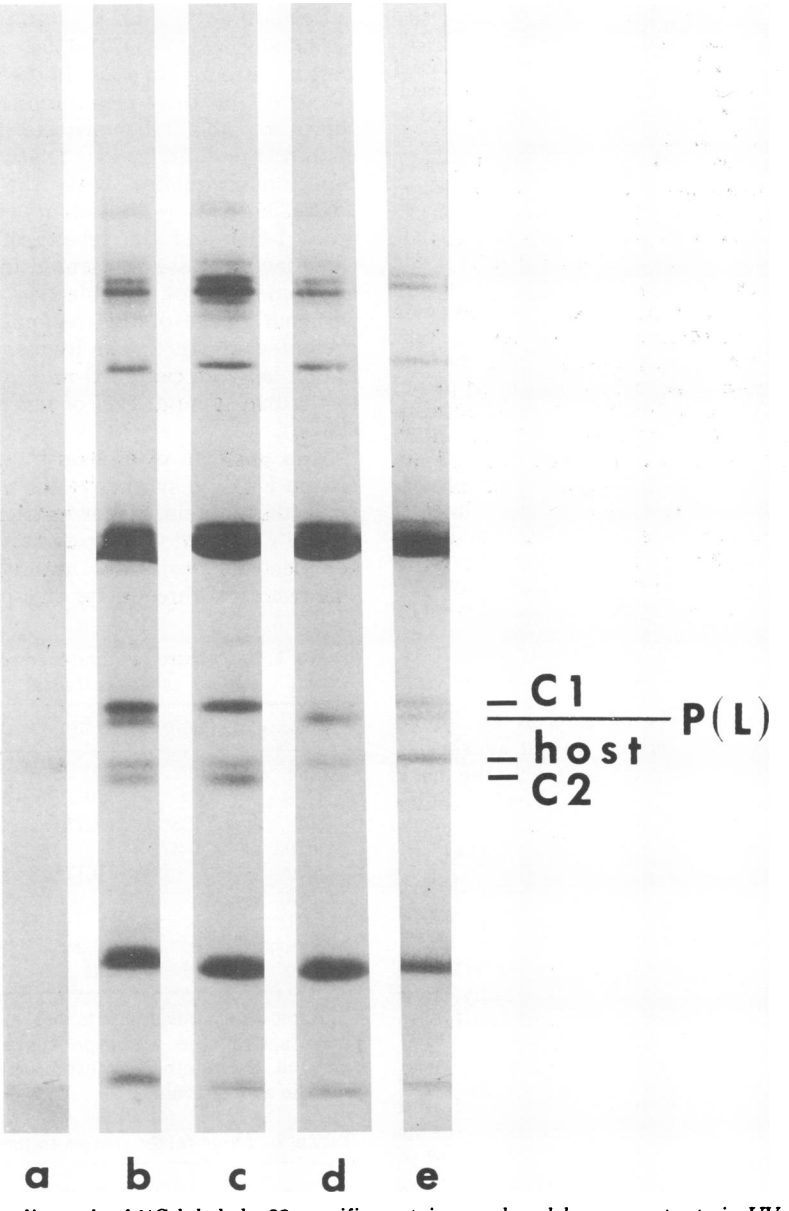

FIG. 1. *Autoradiograph of ^{14}C-labeled φ29-specific proteins produced by sus mutants in UV-irradiated B. subtilis SpoA12. Cells were concentrated to 2 × 10^9/ml, irradiated as described previously (9), and then infected with wild-type φ29 or sus mutants (multiplicity of infection of 50). After 5 min at 37 C the infected cells were diluted 10-fold with prewarmed M40 medium containing 10 μCi of a ^{14}C-labeled amino acid mixture per ml and incubated at 37 C. Labeling was terminated in the wild-type infected and uninfected control cultures at 45 min (9) and at 110 min in the mutant-infected cultures. ^{14}C-labeled viral proteins in supernatant and pellet fractions of the infected cultures were processed and analyzed by sodium dodecyl sulfate electrophoresis on a 10 to 20% linear polyacrylamide gradient as described previously (9). Profiles a and b represent supernatant fractions of the uninfected control and wild-type infected cultures, respectively. Profiles c, d, and e are of supernatant fractions of mutant-infected cultures: c, L300; d, I302; e, 0683.*

type revertants of each cistron J mutant were tested by the toothpick method and all gave turbid plaques. If both parents gave a clear plaque but had mutation in different cistrons, a random sample of 204 plaques was examined by qualitative complementation. The proportion of the clear parent in the burst was inferred from the pattern of complementation.

Labeling and electrophoresis of φ29 proteins. φ29

proteins were labeled with a mixture of ^{14}C-labeled amino acids (New England Nuclear Corp; NEC 445, about 200 mCi/mmol, 10 μCi/ml) after infection of UV-irradiated *B. subtilis* SpoA12 as described previously (9). ^{14}C-labeled ϕ29 proteins were prepared in infections of the *B. subtilis* suppressor strain L15 as follows: cells grown in M40 medium supplemented with 100 μg of Casamino Acids per ml were collected by centrifugation, resuspended to a concentration of 2 $\times$ 10^9 cells/ml in M40 medium containing only tryptophan (50 μg/ml), and UV irradiated for 4 min as described previously (9). After infection at 36 C with a multiplicity of infection of 50, the cultures were incubated with agitation, and 9 min later the cells were diluted 10-fold with prewarmed M40 medium supplemented with 40 μg of Casamino Acids per ml and containing a mixture of ^{14}C-labeled amino acids (NEC 445, about 200 mCi/mmol, 10 μCi/ml). At 40 min after infection, the cultures were transferred into an equal volume of a chloramphenicol-azide-phenylmethylsulfonylfluoride mixture (9) at 22 C. In all of these experiments the cells were collected and lysed as described previously, and the ^{14}C-labeled proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 12% gels and analyzed by autoradiography (9).

RESULTS

Phenotypic analysis of cistron O. Three mutants placed in cistron I do not synthesize the structural proteins C1 and C2, the neck upper and lower collars, respectively, under nonpermissive conditions (4). However, when additional mutants placed in this cistron by the relatively insensitive method of qualitative complementation were examined for synthesis of ^{14}C-labeled viral proteins in restrictive infections by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography, four mutants were identified that could synthesize protein C1, the neck upper collar, but not protein C2, the neck lower collar (Fig. 1). This new cistron has been designated cistron O, and the synthesis of high-molecular-weight proteins by these mutants (Fig. 1e) can be contrasted to protein synthesis after infection by mutants L300 and I302 (Fig. 1c and d).

Complementation of cistron O mutants. The results of intercistronic burst-size complementation of *sus* mutants of cistron O are presented in Table 1. The four mutants of cistron O fail to complement each other (data not shown). A consistent low level complementation with mutant I302 is observed, and cistron O mutants seem to fall in discrete classes when complementation of mutant J305 is measured. These results differ from those obtained with the mutants of cistrons F, H, and K that we employed.

Recombination of O mutants. Recombinants O683-J305, O771-J305, and O683-I302 were constructed to assist in the mapping of the O cistron by three-factor crosses. The results shown in Table 2 demonstrate that each of the cistron O mutants gives a relatively low recombination frequency with the recombinant I302-J305. This is consistent with the cistron order I-O-J and the results of the series of three-factor crosses presented in Table 3. The additional crosses of Table 2 are also consistent with our image of this region of the map as presented in Fig. 2. The frequency of recombination between cistron O mutants is very close to the background level of reversion (data not shown).

Map position of cistron F. *sus* mutants in cistron F fail to synthesize the major head and head fiber proteins under restrictive conditions when ^{14}C-labeled viral proteins are analyzed by gel electrophoresis and autoradiography (4). The results of three-factor crosses that position

TABLE 1. *Intercistronic burst-size complementation of sus mutants*[a]

Mutants	O670	O672	O683	O771
F769	46[3]	32[3]	25[3]	34[3]
H756	42[4]	33[4]	38[4]	34[4]
I302	2.8[4]	1.7[4]	2.2[4]	2.7[4]
O670	0.37[4]			
O672		0.17[4]		
O683			0.20[4]	
O771				0.15[4]
J305	29[4]	6.9[4]	18[4]	9.7[4]
K330	59[3]	40[3]	40[3]	40[3]

[a] Burst-size measurements are expressed as the percentage of the wild-type control. Superscripts represent the numbers of exeriments used to compute the average values.

TABLE 2. *Three-factor crosses to position cistron O*

	Crosses[a]	Recombination frequency	Relative order
1	I302-J305 $\times$ O683	0.14	I-O-J
2	O771	0.18	
3	O672	0.15	
4	O670	0.16	
5	O683-J305 $\times$ I302	1.3	I-O-J
6	O771-J305 $\times$ I302	0.66	
7	H756-I302 $\times$ O670	1.4	H-I-O
8	H756-I302 $\times$ O672	1.2	

[a] Each cross was made in duplicate and the average recombination frequency was tabulated.

cistron F as well as cistron O are presented in Table 3. The results of two-factor crosses are included in Fig. 2 to indicate the relative position of most of the mutants employed in the construction of Table 3.

Phenotype of cistron J mutants. We have isolated five cistron J mutants, each on a separate occasion. Some aspects of the phenotypes of mutants J305 and J602 have been described (4). Lysates of mutant J602 prepared with the nonpermissive host *B. subtilis* SpoA12 have serum blocking power absent in comparable J305 lysates (20).

In Fig. 3 we present an autoradiograph of ^{14}C-labeled φ29 proteins produced by cistron J mutant infection of the suppressor strain *B. subtilis* L15. The profile of φ29c-infected cells (Fig. 3h) illustrates the accumulation of the uncleaved appendage precursor protein P(J) that we have observed after infection of the nonpermissive host strain (4, 9). With *B. subtilis* L15 suppression appears to be limited. Apparent fragments of protein P(J) are visible in profiles of mutants J602, J662, and J874 (Fig. 3b, c, and e).

Genetic analysis of cistron J. The results of two-factor crosses shown in Fig. 2 indicate about 1% recombination between mutants J305 and J662. We have constructed recombinants with the clear plaque morphology of the spontaneous mutant φ29c using our reference *sus* mutants (18). The data obtained by three-factor crosses presented in Table 4 is consistent with the order I302-J305-c-K330. From Table 5 we infer the map order J662-c-K330. When the recombinant J305-c is crossed with the mutants J602, J662, or J874 all of the progeny phage have the clear plaque morphology. We cannot demonstrate that the parental genotypes are present in the progeny phage in the proper proportions but the data suggest the map sequence J305-J662-c-K330.

TABLE 3. *Results of three-factor crosses*

sus markers			No. of tests	Recombination frequency[a]		
1	2	3		1 × 23	2 × 13	3 × 12
E626	G614	F769	5	1.4	0.35	1.8
G614	F769	H756	6	1.0	0.44	5.2
F769	H756	I302	3	3.1	0.56	1.6
H756	I302	O683	4	3.2	0.09	0.95
I302	O683	J305	4	1.0	0.10	4.4
O683	J305	K330	4	2.6	0.24	3.1

[a] The central mutant is listed in column 2, thus 1-2-3 order is the left to right cistron order.

DISCUSSION

The genes that code for the φ29 structural proteins that we can identify (9) cluster on the map as depicted in Fig. 2 and 4. Mutants of cistron I fail to synthesize the neck collar proteins previously termed NP2 and NP3 (12) or C1 and C2 (9). We have conventionally named the product of cistron X,P(X), when a single polypeptide was absent from the gel profile of a restrictive mutant infection. Since mutation in cistron O results in the absence of the structural protein named C2 or NP3 from the gel profile, we now name the protein P(O).

During our analysis of *sus* mutant infection of the nonpermssive host we have frequently detected the presence of new polypeptides that are probably fragments of the normal gene product (4). Camacho et al. (5) have presented additional evidence that the suppressor we employ (8) is a suppressor of nonsense mutation. The genetic results of Moreno et al. (13) with mutants of their cistrons E and H (our cistrons I and O, respectively, from joint unpublished data) are interpreted to indicate a strong polarity effect on translation of the mRNA transcribed from the collar region of the genome in the restrictive host. We have confirmed the results of their quantitative complementation experiments using mutants from both collections (unpublished data) and provisionally suggest that the protein C1 or NP2 is the sole product of cistron I and could be named P(I).

Our interpretation of the results of McGuire

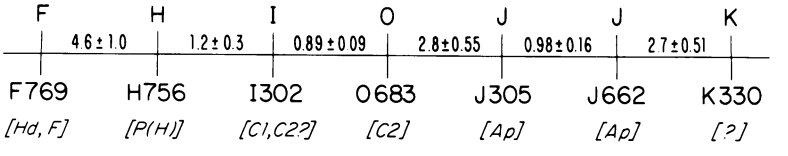

FIG. 2. *A portion of the genetic map of phage φ29. Cistron letters appear above the line; mutants used are below. Values placed on the line are recombination frequencies for two-factor crosses between adjacent mutants. Five or more experiments were used to compute the means and the standard deviations. Cistron order was determined by three-factor crosses. The symbols in brackets refer to the viral proteins described previously (4, 9) and reflect our current understanding of cistron function (4).*

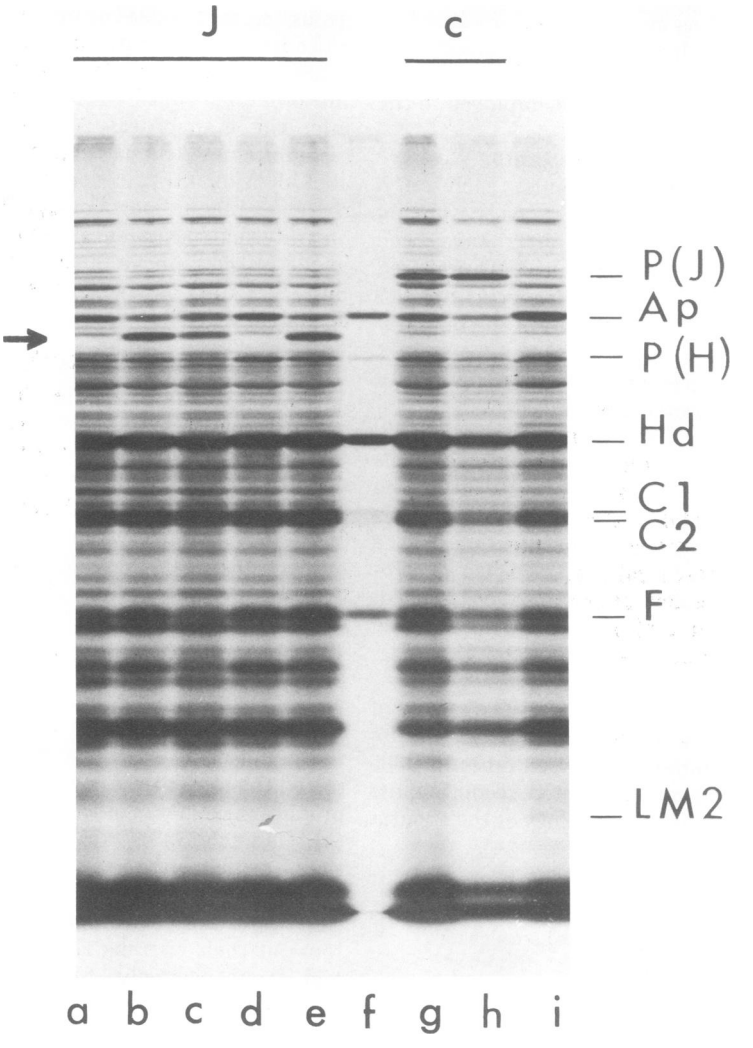

FIG. 3. *Autoradiograph of ^{14}C-labeled ϕ29 proteins produced by gene J sus mutants in infection of the suppressor strain B. subtilis L15. Cells grown in M40 medium supplemented with 100 μg of Casamino Acids per ml were resuspended in unsupplemented M40 medium and UV-irradiated for 4 min as described. After infection with a multiplicity of infection of 50, the cultures were incubated at 36 C with agitation, and 9 min later the infected cultures were diluted 10-fold to 2 $\times$ 10^8 cells/ml with prewarmed M40 supplemented with 40 μg of Casamino Acids per ml and containing a mixture of ^{14}C-labeled amino acids (10 μCi/ml). At 40 min after infection, the cultures were transferred into an equal volume of a chloramphenicol-azide-phenylmethylsulfonyl-fluoride mixture (9) at 22 C. The cells were collected by centrifugation and treated as described previously (9). The pellet fractions were analyzed by sodium dodecyl sulfate electrophoresis. Profiles of the mutant infections and purified ϕ29 virions a through i, respectively, are: (a) J305; (b) J602; (c) J662; (d) J716; (e) J874; (f) purified ϕ29 virions; (g) M741c; (h) ϕ29c; (i) ϕ29 wild type. The arrow indicates the position of P(J) fragments.*

et al. (11) is that cistrons IV, V, and VI that cluster in the center of their ϕ29 map correspond to our cistrons H, I, and J, respectively, in the same order.

sus mutants in cistron F fail to synthesize the major head and head fiber proteins under restrictive conditions (4). The data presented in Table 3 support the map position for cistron F presented in Fig. 2 and 4. The synthesis of both major head and head fiber proteins seems to occur in normal amounts at the nonpermissive temperature with temperature-sensitive mutants in cistron F (unpublished data). We are testing the hypothesis that the results obtained with cistron F are analogous to our observations with mutants of cistron I.

TABLE 4. *Results of three-factor crosses to position the clear (c) mutation*[a]

Parental genotypes	Recombination frequencies[b]	Progeny phage φ29		
		Parents (% clear)	Recombinants	
			No.[c]	Clear (%)
I302 + c / + J305 +	4.2	47	204	93
	4.0	45	204	92
I302 + + / + J305 c	5.0	38	204	4
	5.0	62	204	4
J305 c + / + + K330	3.8	53	612	49
	6.0	53	646	53
J305 + + / + c K330	2.6	40	714	35
	3.4	53	612	33
O670 + + / + J305 c	3.3	51	204	3
	4.9	47	204	8
O683 + + / + J305 c	3.0	57	204	3
	3.5	50	204	5
O670 + c / + J305 +	3.1	42	204	93
	4.0	51	204	92

[a] Each cross was performed in duplicate.
[b] Values were determined by methods employed previously (18).
[c] Number of recombinant phage chosen at random to be tested for the clear marker.

The recombination frequency of 4.6 ± 1.0% between mutants F769 and H756 obtained by two-factor crosses suggests that there is a rather large unmarked interval between these mutants. There may be cistrons that code for other structural proteins in this interval. We have never detected one candidate, the minor head protein described by Mendez et al. (12), when UV-irradiated cells were infected under restrictive conditions and when ^{14}C-labeled viral proteins were analyzed by gel electrophoresis and autoradiography (4, 9). Nor have we been able to identify the protein or to obtain mutants in the cistron that codes for the protein that circularizes the DNA molecule in the virion as described by Ortin et al. (14). Nevertheless we must continue to isolate mutants and refine our electrophoretic analysis in the event that these structural proteins exist as independent entities and that the cistrons that code for these proteins are in the F-H interval of our map.

We have reported evidence that the product of cistron J, P(J), is cleaved to generate the appendage protein Ap and low-molecular-weight product LM2 and that infection by the spontaneous mutant φ29c leads to the accumulation of P(J). Carrascosa et al. (6) have confirmed these results with pulse-chase experiments and indicate that the proteins we call P(J) and the appendage Ap have a very similar peptide composition as revealed by tryptic peptide analysis. The low level of suppression illustrated in Fig. 3 is also consistent with the observation of Camacho et al. (5).

We have established that the clear point mutation maps very close to cistron J, but we have not proven that it is in cistron J. The mutants J602, J662, and J874 all produce proteins presumed to be fragments of P(J) that are slightly smaller than the structural appendage protein Ap, and recombination within this group of mutants occurs at a low frequency (data not shown). By contrast, the mutants J305 and J716 do not appear to yield fragments, and we cannot measure recombination between them (data not shown).

TABLE 5. *Results of three-factor crosses to position the clear (c) mutation*

Parental genotypes	Recombination frequencies[a]	Progeny phage φ29		
		Parents (% clear)[b]	Recombinants	
			No.[c]	Clear (%)
J662 + + / + c K330	2.4[d]	49	816	56
	2.3	49	568	59
+ J662 + / J305 + c	1.0	—[e]	408	96
+ J602 + / J305 + c	0.88	—	204	96
+ J874 + / J305 + c	0.94	—	204	95

[a] Values were determined by methods reported previously (18).
[b] The yield of the clear parent was determined by qualitative complementation and employed 204 plaques chosen at random.
[c] Number of recombinant phage chosen at random to be tested for the clear marker.
[d] This cross was performed in duplicate.
[e] We were unable to determine the parental yield by either complementation or clear plaque phenotype.

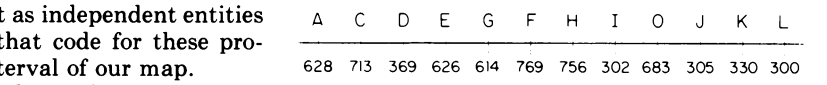

FIG. 4. *This genetic map of φ29 sus mutants merely reflects cistron order. Cistron letters appear above the solid line; mutants used are below. Recombination frequencies are not implied.*

These results have led to the hypothesis that cistrons I, O, and J are transcribed from the heavy strand of the DNA from left to right with orientation to our map (Fig. 4). This is consistent with our mapping within cistron J, the presence of the protein fragments, and the genetic results of Moreno et al. (13) that indicate polarity in translation of mRNA encoded by this region of the genome during infection of the restrictive host by *sus* mutants. The description of P(I) and P(O) presented here, the data on the temporal synthesis of proteins (9), and the heavy strand transcription data of Schachtele et al. (19) are also in agreement. We have constructed recombinant phage to determine if the apparent polarity of translation extends into cistron J.

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LITERATURE CITED

1. Adams, M. 1959. Bacteriophages. John Wiley and Sons Inc., New York.
2. Anderson, D. L., D. D. Hickman, and B. E. Reilly. 1966. Structure of *Bacillus subtilis* bacteriophage ϕ29 and the length of ϕ29 deoxyribonucleic acid. J. Bacteriol. **91**:2081-2089.
3. Anderson, D. L., and E. T. Mosharrafa. 1968. Physical and biological properties of phage ϕ29 deoxyribonucleic acid. J. Virol. **2**:1185-1190.
4. Anderson, D. L., and B. E. Reilly. 1974. Analysis of bacteriophage ϕ29 gene function: protein synthesis in suppressor-sensitive mutant infection of *Bacillus subtilis*. J. Virol. **13**:211-221.
5. Camacho, A., E. Moreno, J. L. Carrascosa, E. Vinuela, and M. Salas. 1974. A suppressor of nonsense mutations in *Bacillus subtilis*. Eur. J. Biochem. **47**:199-205.
6. Carrascosa, J. L., A. Camacho, E. Vinuela, and M. Salas. 1974. A precursor of the neck appendage protein of *B. subtilis* phage ϕ29. FEBS Lett. **44**:317-321.
7. Carrascosa, J. L., E. Vinuela, and M. Salas. 1973. Proteins induced in *Bacillus subtilis* infected with bacteriophage ϕ29. Virology **56**:291-299.
8. Georgopolous, C. P. 1969. Suppressor system in *Bacillus subtilis* 168. J. Bacteriol. **97**:1397-1402.
9. Hawley, L. A., B. E. Reilly, E. W. Hagen, and D. L. Anderson. 1973. Viral protein synthesis in bacteriophage ϕ29 infected *Bacillus subtilis*. J. Virol. **12**:1149-1159.
10. Loskutoff, D. J., and J. J. Pene. 1973. Gene expression during the development of *Bacillus subtilis* bacteriophage ϕ29. II. Resolution of viral-specific ribonucleic acid molecules. J. Virol. **11**:87-97.
11. McGuire, J. C., J. J. Pene, and J. Barrow-Carraway. 1974. Gene expression during the development of bacteriophage ϕ29. III. Analysis of viral-specific protein synthesis with suppressible mutants. J. Virol. **13**:690-698.
12. Mendez, E., G. Ramirez, M. Salas, and E. Vinuela. 1971. Structural proteins of bacteriophage ϕ29. Virology **45**:567-576.
13. Moreno, F., A. Camacho, E. Vinuela, and M. Salas. 1974. Suppressor-sensitive mutants and genetic map of *Bacillus subtilis* bacteriophage ϕ29. Virology **62**:1-16.
14. Ortin, J., C. Vasquez, E. Vinuela, and M. Salas. 1972. DNA-protein complex in circular DNA from phage ϕ29. Nature (London) New Biol. **234**:275-277.
15. Pene, J. J., P. C. Murr, and J. Barrow-Carraway. 1973. Synthesis of bacteriophage ϕ29 proteins in *Bacillus subtilis*. J. Virol. **12**:61-67.
16. Polsinelli, M., and M. Beretta. 1966. Genetic recombination in crosses between *Streptomyces aurefaciens* and *Streptomyces rimosus*. J. Bacteriol. **91**:63-68.
17. Reilly, B. E., and J. Spizizen. 1965. Bacteriophage deoxyribonucleate infection of competent *Bacillus subtilis*. J. Bacteriol. **89**:782-790.
18. Reilly, B. E., V. M. Zeece, and D. L. Anderson. 1973. A genetic study of suppressor-sensitive mutants of the *Bacillus subtilis* bacteriophage ϕ29. J. Virol. **11**:756-760.
19. Schachtele, C. F., C. V. DeSain, and D. L. Anderson. 1973. Transcription during the development of bacteriophage ϕ29: definition of "early" and "late" ϕ29 ribonucleic acid. J. Virol. **11**:9-16.
20. Tosi, M., and D. L. Anderson. 1973. Antigenic properties of bacteriophage ϕ29 structural proteins. J. Virology **12**:1548-1559.