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The Genome of *B. subtilis* Phage SPP1

Physical Arrangement of Phage Genes

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Summary. 41 genes of SPP1 have been delineated by using complementation analyses of 75 conditionally lethal (*ts* and *sus*) mutations. The physical locations of these genes on the SPP1 chromosome have been determined by transfection/marker rescue experiments in which restriction endonuclease generated fragements of SPP1 DNA were used as donor DNA. The physical order of these fragments has been previously established (Ratcliff et al., 1979).

Introduction

We have previously described experiments which led to the physical characterization of the genome of SPP1 (Morelli et al., 1978; Morelli et al., 1979; Ratcliff et al., 1979). The DNA of SPP1 has a molecular weight of 28.6 Mdal, the DNA is partially circularly permuted and terminally redundant. The same conclusions were reached by McIntosh et al. (1978). Many SPP1 mutants, including plaque type mutants and conditionally lethal suppressor and temperature sensitive mutants, have been isolated. These mutations define approximately 50 cistrons of SPP1, and preliminary determinations of gene arrangements by phage crosses have been reported (Spatz and Trautner, 1970; Burger and Trautner, 1978). Some SPP1 genes have been characterized in terms of the proteins or functions which they affect (Esche et al., 1975; Burger et al., 1978; Mertens et al., 1979). Part of our physical studies of the SPP1 genome established the order of restriction fragments generated by a number of restriction endonucleases (Ratcliff et al., 1979). Genetic markers can be rescued from such fragments in a situation where competent *B. subtilis* cells were infected with a conditionally lethal phage, exposed to isolated SPP1 WT restriction endonuclease generated fragments, and then plated under conditions non-permissive for the growth of the mutant phage. Using this technique we have determined the physical arrangement of the available SPP1 genes. The same technique has been used by Cregg and Stewart (1978) to localize genes on the chromosome of *B. subtilis* phage SPO1.

* Part of this work is from the doctoral thesis submitted by M. Behncke to the Freie Universität Berlin (1973).
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Materials and Methods [1]

Plates, Media and *Buffers* used in these experiments were described in previous publications (Biswal et al., 1967; Rottländer and Trautner, 1970; Esche et al., 1975, Ratcliff et al., 1979).

Bacteria. The strains of *B. subtilis* used in this study are described in Table 1.

Bacteriophage. SPP1 WT was from our collection. Phage mutants isolated in our laboratories are mentioned under "Results". Several hydroxylamine induced *ts*-mutants of SPP1 were kindly made available to us by Professor M. Polsinelli of the University of Florence. These mutants are designated by the prefix "I".

Mutagenesis and Isolation of Mutants. SPP1 WT stocks were either mutagenized by treatment with hydroxylamine (Spatz and Trautner, 1970) or (for *ts*-mutants) by growth of SPP1 WT in a mutator strain of *B. subtilis* (Montenegro et al., 1976). ts-mutants designated by the prefix A were made using N-methyl-nitrosoguanidine treatment of SPP1 WT infected cells according to Cerdá-Olmeda et al. (1968). Temperature sensitive (*ts*) mutants were identified by plating the mutagenized phage stocks at 30° and then replica plating the plaques obtained on plates at 46°. A similar technique was used to identify suppressor sensitive (*sus*) mutants: the mutagenized stock was first plated with the permissive indicator from which plaques were replica plated onto plates seeded with the nonpermissive strain. Only mutations which reverted to WT at a frequency of less then 1 in 10^4 were used in this study.

Growth of Mutant Phage Stocks

ts-Mutants. A fresh single plaque was suspended in 1 ml of TY broth and the contents spread on 6 LTT plates with MCB plating

[1] *Abbreviations used*: pfu = plaque forming units; *sus* = suppressor sensitive; *ts* = temperature sensitive; WT = wild type

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Table 1. Bacterial strains used (all strains are derivatives of *B subtilis* 168)

Strain	Genotype	Purpose in this investigation	Reference
MCB	*trp* C2	Growth of *sus*$^+$ phage stocks, plating bacteria	Spatz and Trautner (1971)
MCB su3$^+$	*leu sus*3$^+$	Plating bacteria for *sus* phages	Behncke (1973)
HA101B	[*his met leu*] su$^+$ *try* C2	Growth of *sus* phage stocks	Okubo and Yanagida (1968)
222	*arg trp* C2	Transfection experiments	Trautner et al. (1974)
BD337	*trp*C2 *thr*5 *mut*1	Production of mutants	D. Dubnau quoted in Montenegro et al (1976)

bacteria. After overnight incubation at 30°, leading to confluent lysis, the top layer of each plate was collected with 1 ml of TBT. The scrapings of the plates were pooled, centrifuged at low speed to remove debris and unlysed cells, and then millipore filtered (∅ 0.45 μ). Stocks gave titers of $\sim 2 \times 10^{10}$.

sus-Mutants. HA101B cells were grown overnight in MIII medium with the addition of 50 μg tryptophane/ml. The cells were collected by centrifugation, resuspended in the same volume of NB medium, and incubated at 37°. A single isolated *sus* mutant plaque from an overnight plate was inoculated into 100 ml of such bacteria. Lysis of the culture was observed after approximately 7 h at 37°. These crude lysates contained 10^9–10^{10} pfu/ml, reversion frequencies were between 10^{-6} and 10^{-5}.

Purification of Lysates. SPP1 lysates were concentrated by centrifugation and subsequently purified by sedimentation through a preformed CsCl step gradient ($\rho = 1.7$; 1.5; 1.3 g/ml). Centrifugation was in a Spinco SW41 rotor for 150 min at 28 000 rpm. Phages accumulating in a sharp band within the central layer were collected, extensively dialyzed against TBT and stored at 4°.

Complementation Tests. Equal volumes of conditional lethal mutants were combined at a concentration of 10^7 pfu/ml each in wells of microtiter plates. The mixtures were spotted on TY plates seeded with MCB. Plates were incubated overnight at 46°. Clearing of the spotted region in *ts* × *ts*, *ts* × *sus*, and *sus* × *sus* mutant combinations indicated complementation. As a control, all mutants in each test were also spotted alone.

Restriction Enzyme Digestions and Isolation of DNA Fragments. Restriction endonuclease digests of SPP1 DNA were performed according to the conditions given by the commercial suppliers. Endonuclease generated fragments were separated by vertical slab gel electrophoresis in the presence of 0.5 μg ethidium bromide/ml (Ratcliff et al., 1979). At least three aliquots of a digest were subjected to electrophoresis in adjacent positions. The outside tracks were photographed to identify band positions, whilst DNA was isolated from the central track(s) which had been shielded during the long wave UV irradiation required for photography. Agarose pieces, containing the desired DNA fragments, were cut out from the central track(s), washed extensively with 5 mM TRIS/HCl (ph 7.5) and DNA was extracted from them by the freeze/squeeze method of Thuring et al. (1975). EtBr, still present in isolated DNA fragments, did not interfere with either subsequent restriction endonuclease digestions of individual fragments or with marker rescue. The purified DNA fragments were stored at 4° and remained biologically active for many months after isolation.

Marker Rescue Experiments. In these experiments, 222 cells grown to competence according to Rottländer and Trautner (1970), were incubated for 10 min at 30° in the presence of 0.1 M $MgCl_2$. These cells were distributed into 0.5 ml aliquots (Eppendorf centrifuge tubes) to which were added small volumes of concentrated phage to give an input multiplicity between 1 and 5 pfu per cell. Adsorption was allowed for 10 min at 37°. Equal volumes of these helper infected cells (HIC) (between 10 and 30 μl) and extracted DNA were mixed and incubated for 30 min at 37° The DNA uptake reaction was stopped by placing the reaction tubes at 0° and by adding 200 μl of an overnight culture of indicator MCB cells. Aliquots of 20 μl from this tube and/or from appropriate dilutions were plated on LTT plates When helper phages of the *sus* type were used the plates were incubated at 37°. Incubation at 46° was required if *ts* helper phages were employed. Control experiments, run with each experiment were i) determination of the revertant background in HICs in the absence of added DNA and ii) determination of the helping capacity of a particular helper. The latter parameter was assayed by adding undegraded SPP1 WT DNA at a concentration of 5×10^{-3} μg/ml to the HICs. No plaques were detectable at this DNA concentration in 20 μl of the transfection mixture with *uninjected* competent cells, whereas large numbers of infective centers were produced with HICs. A marker was assigned to a particular endonuclease generated fragment if the marker rescue in a marker/fragment combination exceeded the background by at least a factor of twenty. This *positive* assignment of a gene to a particular fragment was supported by the *absence* of marker rescue in combinations with other biologically active fragments of the particular digest under investigation. Recombination studies with phage mutants which had preceeded these analyses allowed us to make rough predictions of the location of a given mutation. Hence in many cases not all possible fragment/mutant combinations were analyzed.

Restriction Endonucleases used and their suppliers were those described by Ratcliff et al. (1979). An additional restriction enzyme, *Xba*I, was purchased from Biolabs (Bethesda, Md.) and used as suggested by the supplier.

Results and Discussion

1. Complementation Analysis

41 genes of SPP1 were characterized by complementation tests between the 75 conditionally lethal mutants isolated. The results of these tests are summarized in Table 2. The genes thus characterized are numbered 1 through 41, from the right end of the map to the left, i.e. starting with the gene closest to the pac site (see Fig. 1).

It is obvious from Table 2 that there is a wide fluctuation in the numbers of mutations which characterize an SPP1 gene. A combination of the effects of gene size, mutagen specificity, hot spots of mutation and our selection of mutation with a low reversion frequency, is probably responsible for this fluctuation. The number of genes mentioned here therefore represents a minimum estimate of the total number of SPP1 genes. A more reliable approximation of

Table 2. Gene assignments

Gene No.	*ts* mutants	*sus* mutants	*Hin*dIII	*Kpn*I	*Kpn*I *Sal*I	*Sal*I	*Sma*I	*Sma*I *Eco*RI	*Eco*RI	*Eco*RI *Xba*I	*Bgl*I
1		2, 87, 106, 114, 119	1	1		1	2+4	2	1		
2		62					2+4		1		
3		19	1	1			2+4		1		
4	M10		1	1		1+2	2+4		12		
5		86		1		1	2+4		9		
6		100, 115, 118, 126, 129	5b	1	1		2+4		9		
7		70					4		9		
8		7	4b	2b	3		2+4	4	3		
9		61, 64, 92, 98, 102, 112, 122, 124, 131		2b	3	1	1		3		
10		32					1				1
11	bM, 2M, 170M		4b	2b	1				3		
12	M20, G19				3	1	1		3		
13		117, 128	4b	2b	3		1		3		
14		31		2b	3	1	1		3		
15	A12								3		
16	M5	82	5a	2b	3	1	1		6		
17		666		2b					6		
18		46		2b	3		1		6		
19	G6						1		6		
20		45							6		
21	I10								6		
22		222		2b					6		
23		76					1		11		1
24		79					1		7		
25	I5, dM							1	2	1	
26	M28			2a			1	1	2		
27	I15, 1M, 5M			2a	2		1	1	2	1	
28	A58			2a	2		1		2	1	
29	3M		3				1	1	2		
30	G7								5		
31	I17			1			3		4		
32	I20F			1		1	3	3	4		
33	B3			1		1	3	3	4		
34		53							1		
35	A40				1				1		
36	I13, I28 A74, A80, A81	12, 109		1		1			1		
37	A14								1		
38	I21, A38, A66	99	1	1	1	1	2	2	1		
39		51							1		
40		22							1		
41	I1, 6M					1	2		1		

The 75 mutants analyzed were assigned to genes 1 through 41 by complementation analyses (Columns 1–3). Columns 4–12 give the fragment numbers of the restriction endonuclease digests indicated to which a gene was assigned on the basis of marker rescue experiments.

the number of SPP1 genes is given by the identification of SPP1 encoded polypeptides. 44 such polypeptides, representing 84% of the coding capacity of SPP1, have been identified (Mertens, Amann and Reeve, 1979). Some of these peptides, and also of those identified by Esche et al. (1975) in in vivo analyses could be assigned to genes described here.

We do not know whether the *sus* mutants isolated here are of the "amber" type. We can, however, distinguish *two* types of *sus*-mutants. The majority of mutants selected on strain HA101B will also plate on the strain characterized by the suppressor su^{3+}, originally described by Georgopoulos (1969), but this is not the case for mutants *sus*222, *sus*666, *sus*19, *sus*70.

2. *Ordering of SPP1 Genes*

We have determined the position of SPP1 genes on restriction endonuclease generated fragments of SPP1

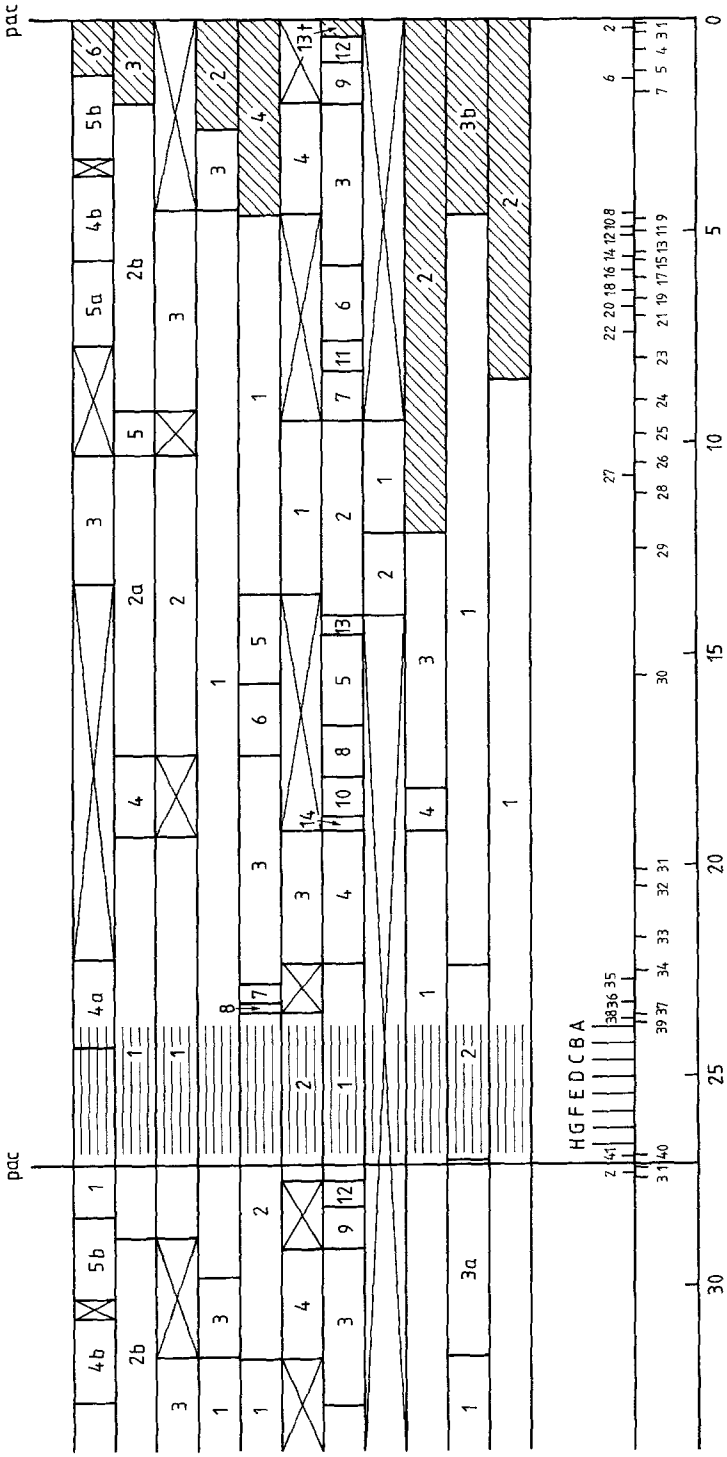

Fig. 1. Restriction Map of SPP1. This map corresponds to the restriction map previously published (Ratcliff et al., 1979) with modifications as mentioned in the text. We have also entered the positions of selected fragments obtained by double digests and have included on the basis of their gene content positions of *Hin*dIII fragments. The line indicated as "Gene No." shows the arrangement of genes as derived from the marker rescue experiments of Table 2 and genetic crosses (Lüder and Trautner, unpublished). The shaded area in the left part of the map describes a dispensable region of the SPP1 chromosome, as defined by the occurrence of deletion mutants. Mutations in this region are indicated by capital letters. Composite fragments of multiple bands are indicated by small letters behind the fragment number

DNA by marker rescue techniques. The physical sequence of fragments has been previously determined (Ratcliff et al., 1979). The use of various restriction enzymes for such studies which produce overlapping restriction fragments increased the resolution of the analyses. Assigments of genes to individual fragments of double or triple bands were made by comparing the marker rescues with the DNA at the position of the isolated double band before and after digestions with a second restriction enzyme, which would eliminate one or two bands from the composite band. Results from the two factor crosses between certain pairs of markers (Lüder and Trautner, unpublished data) were used in some cases to determine the orientation of markers within fragments.

The Restriction Map. The physical map of the SPP1 genome (Ratcliff et al., 1979) provided the basis for our mapping experiments. We have adjusted this map in three regions (Fig. 1). (1) Using *Eco*RI partial digestion of *Sal*I fragment 2, the unknown order of *Eco*RI fragments in the terminal region has now been determined to be 3–9–12–13t–*pac*. (2) We have identified the *pac* terminal "minor" band 13t of the *Eco*RI digest by using polyacrylamide gel electrophoresis which demonstrated that band 13t has a molecular weight of 0.4 Mdal and is hence slightly smaller than band 13 (4.4 Mdal). These bands were not resolved before. (3) Additional digestions with the enzyme, *Xba*I, required that we correct our previous fragment order between *Eco*RI fragments 4 and 2, i.e. between positions 19.5 and 14 M daltons, to be ...4–14–10–8–5–13–2... This sequence is similar to that established by McIntosh et al. (1978) who first used *Xba*I to digest SPP1 DNA (4). Fragments 2a, 2b, and 3 of the published *Bgl*I digest were renamed 3a, 2, and 3b in slight correction of the original molecular weights. The same holds for the *Kpn*I digest: fragments 2, 3, 4, 5, 6 were renamed 2a, 2b, 3, 4, 5. We have not yet been able to assign a physical location for *Eco*RI fragment 15 (0.25 Mdal). Insertion of this fragment would lead to slight alterations of some fragment positions.

Marker Rescue Experiments. Assignment of genes to the 16 separable *Eco*RI generated restriction fragments of SPP1 DNA would give the highest resolution of the SPP1 chromosome. We have therefore concentrated on assigning all SPP1 genes to the ensemble of *Eco*RI fragments. We have also used restriction fragments obtained with other restriction enzymes and fragments obtained by digestion with *two* restriction enzymes. In particular through marker rescues with fragments obtained after consecutive digestions of *Eco*RI generated fragments we were able to assign genes to defined regions within *Eco*RI fragments. Comparisons of the marker contents of overlapping fragments from *different* restriction endonuclease digests provided an independent test for the vailidity of the physical arrangement of restriction fragments in the published SPP1 maps. The localization of the major *Hin*dIII fragments, which had not been previously published, became only possible after their marker contents had been established.

All assignments of SPP1 genes to the various restriction fragments are summarized in Table 2 and Fig. 1.

In the interest of clarity of this presentation we do not present the actual data of all fragment/marker combinations which led to the gene assignments of Table 2. However, in order to illustrate the potential of the marker rescue technique, we show in Table 3 the actual plaque numbers counted which led to the assignments of some genes in the left portion of the SPP1 chromosome (Fig. 1).

The distribution of markers along the restriction maps gives an unequivocal and consistent correlation between the restriction maps and the localization of SPP1 genes. It is realized that this presentation provides only a limited resolution given by the number of separated restriction fragments analyzed. Furthermore the finite size of the genes analyzed is not taken into consideration.

An analysis of Fig. 1 reveals that the genes which we have investigated are not evenly distributed over the SPP1 chromosome. The left and right ends of the molecule have a high gene density whereas this is not true of the more central portion of the map between genes 28 and 31. Whether the observed gene distribution is a true reflection of unequal gene density or is a consequence of our mutant selection procedure must await analyses of the gene products encoded by the various portions of the chromosome. This analysis (Mertens et al., 1979) has already revealed that also DNA of the dispensable region, characterized by deletion mutants, codes for eight – obviously dispensable – polypeptides. We have assigned the letters A, B, C, D, E, F, G, H to these genes, whose sequence was derived from the peptide patterns observed in minicells infected with various deletion mutants.

The marker rescue experiments reported fulfill one of the predictions derived from the structure of the SPP1 DNA and its mode of packaging. Namely, markers carried on the *pac* proximal *minor* fragment of a digest should also be rescuable from that *major* fragment of the digest which carries the *pac* site. We have demonstrated this in marker rescue experiments with the minor band of the *Sma*I digest (*Sma*4) which can readily be separated from overlapping bands in

Table 3. Marker rescue experiments

	Gene No.	6	4	3	1	1	1	38	36	36	33	32	31
	Marker on helper phage	*sus*118	*ts*M10	*sus*19	*sus*2	*sus*87	*sus*114	*sus*99	*sus*12	*sus*109	*ts*B3	*ts*I20F	*ts*I17
*Sma*I	Helper background	19	0	15	10	56	2	0	5	21	11	0	3
	Rescue with.												
	Undegraded DNA	~1200	275	940	164	>5000	278	~2000	1096	202	92	118	84
	Fragment 1	>5000	217	2496	530	>5000	>5000	1184	5	19	7		5
	Fragment 2												
	Fragment 3								4	19	283	256	417
	Fragment 4			334									
	Helper background		0		10			3	8	36	11		
	Rescue with				164			~2000	>5000	>5000	92		
	Undegraded DNA		275										
	Complete *Sma*I digest		310		350			~2000	14	69	332		
*Kpn*I	Helper background	4	84	1	79	37	1	2	9	21	4	6	2
	Rescue with												
	Undegraded DNA	509	>5000	~1100	~3000	~3000	~1100	~2200	~3000	~3000	936	107	736
	Fragment 1	220	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000	960	154	832
	Fragment 2a and 2b	6			96	180	135	347	266	319	18		
	Fragment 3 and 4	12	67		11	30	2	13	19	77	12		
	Fragment 5	8			46	37	5	7	85	808	1		
*Eco*RI	Helper background	0	38	26	79	37	2	1	9	21	10	34	10
	Rescue with:												
	Undegraded DNA	47	~1500	1912	~3000	~3000	278	~1500	~3000	~3000	396	520	646
	Fragment 1			82	~900	~1500	241	~2000	~1500	~1500			
	Fragment 4										2990	1920	4300
	Fragment 9	83											
	Fragment 12		~2000										
*Hin*dIII	Helper background	10	84	19	5	13	10	1	5	20	2	2	
	Rescue with.												
	Undegraded DNA	960	>5000	750	~3000	>5000	750	~4000	~3000	~3000	1760	40	
	Fragment 1	13	>5000	1136	149	>5000	1160		6	26			
	Fragment 2	12			11	15			4	20			
	Fragment 3	23			20	29			8	19	3	0	
	Fragment 4	8			8	24		600	284	383	1	3	
	Fragment 5	191	40		6	73			19	41			

Plaque numbers (from 20 µl of the incubation mixture) counted in marker rescue experiments and in controls which led to the gene assignments of Table 2 and Fig 1. In the case of degradation with *Sma*I we have also entered marker rescue values (plus controls) obtained with an unfractionated digest of SPP1 DNA.

a *Sma*I *Xba*I double digest. From Fig. 1 and Table 2 it is clear that genes 1, 2, 3, 4, 5, 6, (7), 8 are carried on the minor *Sma*I fragment 4 *and* on *Sma*I fragment 2. Completely analogous results were reported by Cregg and Stewart (1978) for SPO1 DNA, a phage with unique ends and terminal redundancy.

The use of the minor bands in marker rescue also allows marker locations to be assigned within *Eco*RI fragment 1. Genes 1, 2, 3 which can be rescued from both *Eco*RI fragment 1 and the minor fragment of the *Sma*I digest must be located between the left end of *Eco*RI fragment 1 and the DNA sequence determining the pac site. The pac site is 0.4 Mdal (size of *Eco*RI fragment 13t) distant from the *Eco*RI cleavage site separating *Eco*RI fragments 1 and 12.

In addition to establishing a physical map of SPP1 genes we have made the following observations: (1) Neither the *molecular ends* of restriction fragments (staggered, flush, or mixed (from double digests)) used in a marker rescue experiment, nor the location of a *marker within such a fragment* with respect to the molecular ends effects the efficiency of marker rescue.

(2) The same is true of the *presence of partial nonhomology* caused by the use of deletion mutants between phage genome and restriction fragment: e.g. the efficiency of marker rescue from SPP1 WT *Eco*RI fragment 1 and from the corresponding fragment isolated from deletion mutants of SPP1 are identical. This finding allowed the use of restriction endonuclease digests of deletion carrying SPP1 DNA in those cases (*Sma*I, *Bgl*I) were the resolution of fragments in electrophoresis could be enhanced by the use of deleted DNA instead of WT DNA. (3) With a few markers we were unable to detect marker rescue after digestion of SPP1 DNA with a restriction enzyme although such markers were readily rescuable *before* enzymatic digestion or following digestion with other restriction enzymes. For example, *sus*109 from gene 36 is readily detectable on fragments from *Eco*RI, *Kpn*I or *Hin*dIII digests, but could not be located on any *Sma*I fragment. Presumably the *Sma*I cut which separates *Sma*I fragments 2 and 7/8 leads to *destruction of the gene.*

The physical ordering of what must be the majority of SPP1 genes presented here raises the question of whether the established marker distribution reflects a meaningful arrangement in terms of regulation of gene expression. In this connection it is of interest to refer to the analysis of Burger and Trautner (1978) who demonstrated that seven genes involved in SPP1 DNA synthesis are located in a contiguous region at the junction of *Eco*RI fragments 1 and 4. In contrast to these genes, which are only described in terms of the function they effect, a few genes have been identified which code for structural proteins of the phage (Esche et al., 1975; Mertens et al., 1979). These genes are also located in distinct regions of the genome. Biochemical studies are in progress to identify the proteins encoded by individual restriction fragments (Amann and Reeve, in preparation), to localize promoter sites in SPP1 DNA and to follow the kinetics and specificity of SPP1 m-RNA (Montenegro, Morelli and Trautner, in preparation). The results of these experiments should provide a coherent picture of the regulatory processes operative in SPP1 development and their relationship to structural arrangement.

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