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Genetics

Bacteriophage φX174: Gene *A* overlaps gene *B*

(mutants/genetic organization/phage proteins)

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ABSTRACT **The map position of several φX174 mutations in the genes *A* and *B* was determined by marker rescue with DNA fragments produced by the restriction enzymes *Hha* I, *Hin*dII, *Hae* III, and *Alu* I. All the gene *B* mutants were found to be located within gene *A*. Genetic complementation and analysis of phage-specific protein synthesis show that, under restrictive conditions, nonsense mutants in gene *A* do not block the synthesis and activity of the B protein and nonsense mutants in gene *B* do not affect the gene *A* function. The map position of the COOH-terminal end of gene *A* was determined using an amber mutant that synthesizes slightly shortened A and A* proteins. It is concluded from these experiments that gene *A* overlaps gene *B* completely (or almost completely) and that the overlap region can be translated in two ways with different reading frames: one frame for the synthesis of the A and A* proteins and another for the synthesis of the B protein.**

Our insight into the genetic structure and organization of the DNA of bacteriophage φX174 has much improved in recent years. Detailed information is available on the location and order of mutants and genes (1–3) and sequence data of the φX DNA are rapidly accumulating (3–5). Elegant DNA sequence work in Sanger's laboratory showed untranslated regions of unknown function (5) and the existence of one gene (*E*) in another gene (*D*), with the two genes translated in different reading frames (3). Still, many aspects of the organization and function of the phage genome are unclear.

In a previous paper (6) we described an amber mutant (*to*8) that was mapped physically within gene *A*, whereas it did not behave like an *A* mutant. This result indicated either a complex genetic organization of this region or that the number of phage genes had to be increased. In this paper we describe a more detailed analysis of this part of the genome. The results show that (*i*) the gene *A* extends itself far into (or even over) the gene *B* and (*ii*) the overlapping DNA sequence is used in two different reading frames, one frame for the synthesis of the A and A* protein and another frame for the B protein.

MATERIALS AND METHODS

Media, methods, and bacterial strains for phage growth, titration, spheroplast infection, and preparation of single-stranded and double-stranded viral DNA have been described (1).

Phage Mutants. *am*33 (*A*), *am*35 (A), *am*18 (*A*), *am*16 (*B*), *ts*6 (*B*), *ts*116 (*B*), *och*6 (*C*), and *am*6 were given to us by R. L. Sinsheimer (2); *am*S29 (*A*), *ts*56 (*A*), *ts*173 (*B*), and *am*H210 (*B*) were gifts from M. Hayashi (7); *to*8 (*B*), *ts*R5-2 (*B*), and *am*R8-1 were isolated in this laboratory (6).

Isolation of DNA Fragments. The preparation of the restriction enzymes together with their conditions for DNA digestion has been described for *Hin*dII from *Haemophilus influenzae* Rd (R fragments), *Hae* III from *Haemophilus aegyptius* (Z fragments), and *Alu* I from *Arthrobacter luteus* (A fragments) by Vereyken *et al.* (8) and for *Hha* I from *Haemophilus haemolyticus* (H fragments) by Baas *et al.* (9). The RFI digests were fractionated on slab gels. The gels were autoradiographed and the fragments were excised and eluted (8).

Fragment Assay. Restriction fragments were dissolved in 50 mM Tris·HCl (pH 7.0), the circular single-stranded mutant DNA in 0.60 M NaCl/0.06 M sodium citrate, pH 7 (4 × SSC). A 10-μl sample of fragments (A_{260} = 0.05) was added to 10 μl of circular ssDNA (A_{260} = 0.5), heated for 2 min in boiling water, and incubated for 20 min in a 64° water bath. After cooling of the mixture in ice, 0.18 ml distilled water was added. This solution was used in the spheroplast assay.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis. Electrophoresis of cell extracts was performed in 12.5 or 15% slab gels that had a 3% stacking gel (10). Bisacrylamide was used as a crosslinker. Tritium-labeled proteins were made visible by scintillation autography (11); ^{35}S-labeled proteins, by direct autoradiography of dried gels.

Preparation of Infected Cell Extracts. Logarithmic phase *Escherichia coli* HF4704 (nonsuppressor) in TPG medium (12) was pretreated with mitomycin C at 300 μg/ml [to prevent host protein synthesis and cell lysis (13)], washed once with TPG, suspended in pre-warmed TPG medium (4 × 10^8 cells per ml), and aerated for 10 min. This culture (0.5 ml) was incubated at 37° and infected with phage (multiplicity of infection = 5). Twenty-five minutes after infection 50 μCi of [^{3}H]leucine (specific activity 60 Ci/mmol) or 10 μCi of [^{35}S]methionine (specific activity 460 Ci/mmol) was added and the infection was stopped 85 min after infection. The cells were centrifuged, washed, and resuspended in 40 μl of lysis buffer [0.008 M Tris, pH 6.8/4% [sodium dodecyl sulfate, 10% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol]. This solution was kept for 5 min at 100° and then used for gel electrophoresis.

RESULTS

Mutant Mapping. The amber mutant *to*8 that was physically mapped within gene *A* without being an *A* mutant (ref. 6 and Fig. 1) behaved in complementation tests in the same way as the well-characterized *B* amber mutant *am*H210 (14). This made it very likely that *to*8 was in fact a gene *B* mutant. This fact, together with the curious map position, prompted us to do a more detailed analysis of this part of the genome. The previous experiments were extended in two ways: (*i*) by testing more mutants in the fragment assay and (*ii*) by using DNA fragments obtained by cleavage of φX RFI with the restriction enzyme *Hha* I from *Haemophilus haemolyticus* [this enzyme is known to cut twice in this region (9)]. The additional mutants were *am*H210 (*B*), *ts*6 (*B*), *ts*173 (*B*), *ts*R5-2 (*B*), *am*S29 (*A*), and *ts*56 (*A*). It should be pointed out here that we are unable to use *ts*56 (*A*) in the complementation test or to measure its DNA synthesis under restrictive conditions because *ts*56 (*A*) is very leaky under the conditions (41°) where our *ts* mutants are tested. We therefore rely entirely on the genome assignment

Abbreviations: φX, bacteriophage φX174; RF, replicative form DNA (RFI is double-stranded, closed circular); ssDNA, single-stranded DNA.

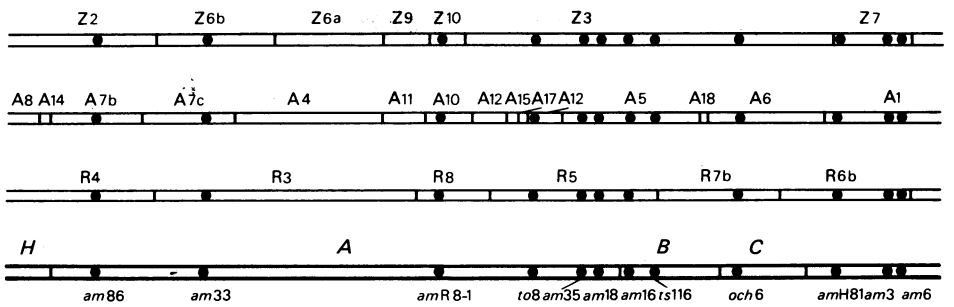

FIG 1. Genetic map (*bottom*) and fragment maps of the gene *A* and gene *B* region. Dots indicate positions of mutations shown on the genetic map.

made by Hayashi and Hayashi (7). Mapping *ts*56 (*A*) is no problem because this can be done at 42°.

All mutants were tested with the various DNA fragments in the fragment assay and the resulting ratio of wild-type phage over total progeny phage (wild type + mutant) for each mutant is given in Table 1. The highest ratio for each set of DNA fragments is used to assign the mutant tested to that particular fragment. All mutations can be put unambiguously in the existing fragment maps, as is shown in Fig. 2. The resulting order of these mutations was, however, rather surprising; going from left to right in Fig. 2 we first find a gene *A* mutation (*am*R8-1), then four B mutations (*to*8, *amH210*, *am*16, and *ts*6) followed by a stretch of DNA of 70 base pairs that contains three *A* mutations (*am*35, *am*18, and *am*S29) and two *B* mutations (*ts*173 and *ts*R5-2). The order of these five mutations is unknown. After this comes *ts*116 (*B*) and finally *ts*56 (*A*). This gives an alternation of *A* and *B* regions in the following way: *-A-B-(AB)-B-A-C*, whereas the distances between the A and B mutations in the H7*a*-R5 fragment overlap must be very small. Therefore, a splitting up of this genome region into separate *A* and *B* genes became rather difficult and we had to consider a more fundamental interaction between the *A* and the *B* gene.

In order to exclude the very small possibility of having interchanged mutant stocks, we tested all the mutants again (except *ts*56, as mentioned before) by genetic complementation and also by measuring their phage-specific DNA synthesis. The *A* mutants did not synthesize progeny RF DNA and complemented like *A* mutants (15), whereas the *B* mutants made normal amounts of progeny RF DNA and complemented like *B* mutants (data not shown).

Protein Synthesis. The amber mutants in this part of the genome were analyzed for their phage-specific protein synthesis under restrictive conditions. HF4704 (nonsuppressor, *uvrA*) cells were treated with mitomycin C (to prevent lysis and to reduce the host protein synthesis) and infected with phage. [^{3}H]Leucine was added 25 min after infection. After washing and lysis the labeled extracts were electrophoresed on 12.5% acrylamide gels. The result after fluorography (11) is given in Fig. 3. As controls, lysates of infections with *am*N1 (*H*) and *am*33 (*A*) and purified phage particles were coelectrophoresed. This identifies the proteins of the genes *A*, *F*, *G*, and *H*. Fig. 3 shows that *am*18 (*A*), *am*S29 (*A*), and *am*35 (*A*) all lack the 55,000- and 36,000-dalton proteins that have been described by Linney and Hayashi (16, 17) as the protein products of gene *A*. The absence of both *A* proteins is in agreement with the map

Table 1. Mapping of the mutants in the fragment assay

Mutant ssDNA		10^3 × ratio of wild-type phage to total phage																	
		*Hin*dII fragments				*Hae* III fragments				*Alu* I fragments					*Hha* I fragments				
		R3	R8	R5	R7*	Z9	Z10	Z3	Z7	A11	A10	A12*	A5	A6	H1	H14	H7	H4	R5A12†
am-R8-1	(*A*)	0.08	9.31	0.01	0.01	0.56	3.6	0.06	0.11	0.03	6.0	0.01	0.02	0.01					
*to*8	(*B*)	0.08	0.02	12	0.03		0.05	15	0.03		0.02	1.2	0.01	0.03	7.4	0.14	0.10	0.01	6.7
am-H210	(*B*)		0.03	5.3	0.01		0.02	6.4	0.01		0.01	1.8	0.01	0.01	3.6	0.01	0.01	0.02	1.8
*am*16	(*B*)		0.33	390	0.75		0.32	25	0.03		0.05	0.03	28	0.63	10	0.32	0.52	0.11	
*ts*6	(*B*)		0.05	1.0	0.01		0.01	8.4	0.01		0.01	0.14	1.6	0.01	0.04	1.8	0.30	0.05	
*am*S29	(*A*)		0.02	1.6	0.01		0.12	17	0.01		0.03	0.02	14	0.07	0.15	0.01	24	0.22	
*am*35	(*A*)		1.5	21	0.57		2.3	110	0.31		0.83	0.76	24	0.99	0.31	0.42	10	0.31	
*am*18	(*A*)		2.3	81	0.01		0.04	39	0.02		0.03	0.02	35	0.17	0.05	0.11	5.4	0.09	
*ts*173	(*B*)		2.1	12	0.01		0.25	7.2	0.19		0.02	0.01	18	0.02	0.01	0.31	61	0.15	
*ts*R5-2	(*B*)		1.8	62	0.11		0.38	220	0.55		0.25	0.20	140	1.6	0.01	0.05	28	0.03	
*ts*56	(*A*)		0.01	0.01	59		0.03	95	0.01		0.01	0.01	42	0.01	0.30	0.18	74	0.23	
*och*6	(*C*)		0.03	0.02	60		0.01	68	0.01		0.05	0.08	0.12	21	0.24	0.30	0.18	23	

The progeny phages obtained after infection of spheroplasts of *E. coli* K58 (suppressor-positive) with the partial duplex DNA were plated under the following conditions: amber (*am*) mutants on HF4712 (UAG suppressor) for total yield (amber + wild type) and on C (nonsuppressor) for wild-type phage, ochre (*och*) mutants on WWU (UAA suppressor) for ochre + wild type and on C (nonsuppressor) for wild type, temperature-sensitive (*ts*) mutants on C at 30° for temperature-sensitive + wild type and on C at 41° for wild-type phage. The rationale of the fragment assay is that when a wild-type DNA fragment covers the mutant allele on the ssDNA, the proportion of wild-type particles in the yield after spheroplast infection will be markedly increased.

* The R7 and A12 preparations contain, respectively, two and four different fragments of the same size.

† The fragment R5A12 is obtained by digesting R5 with *Alu* I and isolating its single intact A12 fragment. A more detailed analysis of *to*8, *am*16, *am*18, *am*35, *ts*116, and *och*6 is given in refs. 1 and 2.

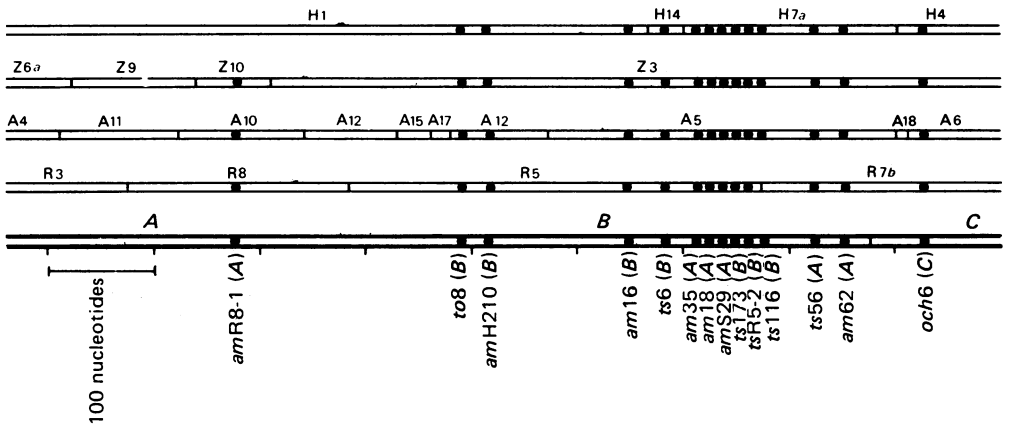

FIG. 2. Integration of the fragment assay results of Table 1 with the physical fragment maps of this part of the DNA. The lowest part of the figure is the resulting genetic map.

position of the mutations. Mutants in the right part of gene *A* do not synthesize the A and A* proteins, whereas mutants in the left part of gene *A* do not synthesize the A protein but make normal amounts of the A* protein (e.g., *am*33). The three *A* mutants, however, synthesize the B protein in about normal amounts. The *B* mutants *am*16, *am*H210, and *to*8 make no B protein but they do synthesize the A and A* proteins. In the case of *am*16 a new protein band appears that is most likely its amber protein fragment. On a 15% gel this protein was found to have a molecular weight of approximately 9000. Given the position of *am*16 on the map of Fig. 3 and the size of the intact B protein (16,500 daltons), an estimate can be made of the start and the end of the *B* gene (see *Discussion*). An interesting point is that the amount of H protein produced seems to be greatly reduced in the infections with the *B* mutants (see Figs. 3 and 5).

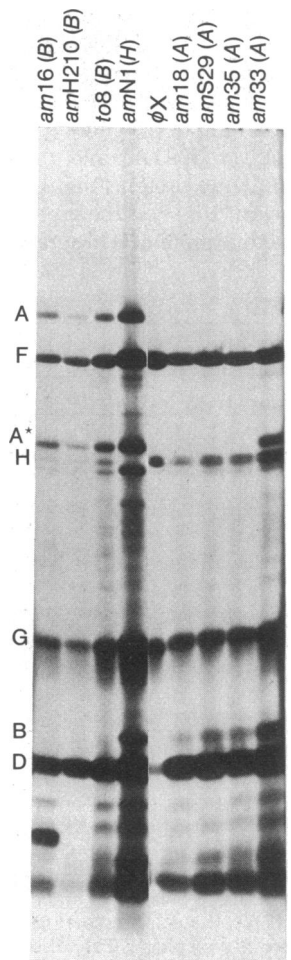

FIG. 3. Autoradiogram of electrophoresis on 12.5% acrylamide gels of ^{3}H-labeled extracts of cells infected with φX mutants. Purified phage particles are co-electrophoresed as protein markers. Migration is downwards.

The conclusion of this protein analysis is that despite their respective map positions, the *B* amber mutants are not blocked in the synthesis of the A and A* proteins and that the *A* amber mutants make a normal-sized B protein.

The COOH-Terminal End of the *A* Gene. The picture that emerges from the experiments described above is that of an *A* gene that extends into or even over the *B* gene, in such a way that there is no translational interference of the two genes. Both genes are translated such that an amber codon in one gene is not seen as a nonsense codon in the other gene (see also Fig. 6).

One of the problems of this model is that although we have a fair idea of the position of the *B* gene from the position of *am*16 and the size of its amber protein fragment, there is no

Table 2. Fragment analysis of *am*6

Fragment used	10^3 × phage ratio C, 37°/HF, 37°	10^3 × phage ratio C, 30°/HF, 37°	Fragments used	10^3 × phage ratio C, 30°/HF, 37°
Z1	0.15	0.14	A1 + R1	1.1
Z2	0.18	0.18	A1 + R2	1.1
Z3	0.25	0.15	A1 + R3	1.4
Z4	0.22	0.15	A1 + R4	1.7
Z5	0.30	0.20	A1 + R5	0.81
Z6*a*	0.35	0.19	A1 + R6	1.5
Z6*b*	0.35	0.18	A1 + R7	14
Z7	1.8	0.21	A1 + R8	2.5
Z8	0.36	0.11	A1 + R9	1.2
Z9	0.18	0.12	A1 + R10	0.74
Z10	0.15	0.16	A1 + A5	11
R1	0.21	0.15	A1 + A6	1.0
R5	0.25	0.18	A1 + A12	1.3
R6	2.2	0.17	A1 + Z3	6.3
R7	0.36	0.12	A1 + Z7	1.1
A1	1.3	0.18	A1 + H1	1.3
A2	0.13	0.13	A1 + H4	0.75
A5	0.11	0.15	A1 + H7a	8.5
A6	0.12	0.12	A1 + H14	1.1

C and HF designate *E. coli* strains C and HF4712.

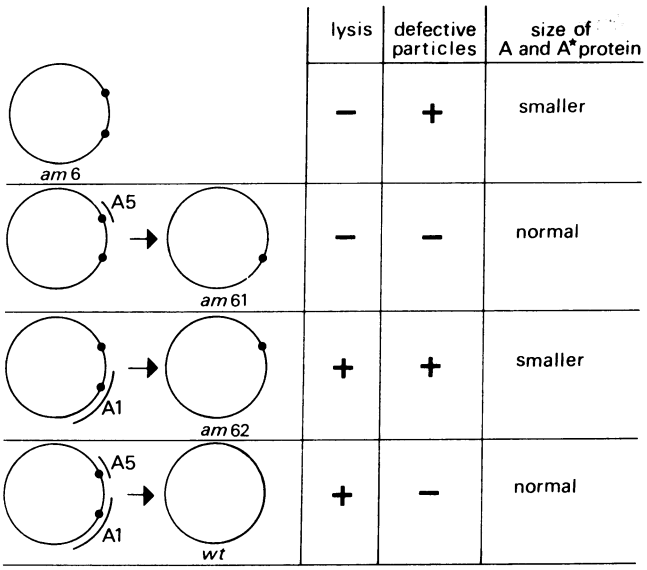

FIG. 4. Genetic composition of *am*6 and its derivatives and their properties in an infection of a nonsuppressor host.

physical evidence as to where on the genetic map the *A* protein ends. Up till now, there has not been found conclusively an A protein fragment from a gene *A* amber mutant, although, for example, with *am*18 at least 90% of the A protein must be made. The protein fragments are either broken down or they block their own synthesis. It could therefore still be that somehow this genome region controls the synthesis of the A protein without being part of the structural gene for this protein. None of our *A* amber mutants produced an A protein fragment, but when we screened our mutant collection for the proteins they specified, we found, to our surprise, a mutant that makes A and A★ proteins which are slightly smaller than their wild-type proteins, the difference being approximately 1000 daltons or about 10 amino acid residues (Fig. 5). This mutant is *am*6, a ϕX mutant with a somewhat unclear genetic history. *am*6 does not lyse the restrictive host and produces in such cells mainly defective particles (18). We had previously mapped *am*6 and found that it was located very close to *am*3 (*E*) and that it was therefore very likely a gene *E* (lysis) mutant (1). Because of the presence of a shortened A and A★ protein, a more elaborate analysis of *am*6 was made. We found that *am*6 contained in fact two amber mutations, one in the lysis (*E*) gene and the other one in the COOH-terminus of the *A* gene. Both mutations were mapped with the fragment assay and the results after hybridization of the DNA fragments (single fragments or a combination of two) to the *am*6 ssDNA and infection of spheroplasts are given in Table 2. When single fragments are used, one observes an increase in the ratio C, 37°/HF, 37° (with the fragments A1, R6, and Z7, see also ref. 1) but not in the C, 30°/HF, 37° ratio. The plaques on C at 37° are rather small but, what is more important, when tested they do not form plaques on C at 30° (this is in contrast to wild-type phage that plates equally well on C at 37° and 30°). Therefore, they are undoubtedly not wild type. Because 37° is our normal temperature for plating of wild-type phages, it is not surprising that *am*6 was originally scored as a single-amber mutant. When all fragments were tested in the presence of fragment A1 (on which *am*6 was mapped previously), we found that A1 in combination with A5, R7, Z3, and H7*a* gives a clear increase in the C, 30°/HF, 37° ratio. Moreover, the plaques on C at 30° plate well on C at 37° and are indistinguishable from wild-type phage. From this we concluded that *am*6 is a double mutant with one mutation on the fragments A1, R6, and Z7 and another mutation on the

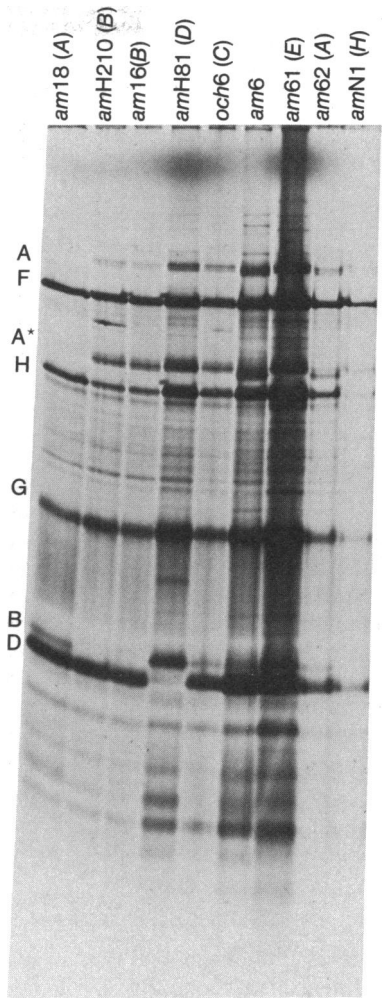

FIG. 5. Autoradiogram of electrophoresis on 15% acrylamide gels of ^{35}S-labeled extracts of cells infected with ϕX mutants.

fragments A5, R7, Z3, and H7*a* [this is between *ts*116 (*B*) and *och*6 (*C*) in Fig. 2]. Both mutations are considered to be amber mutations because *am*6 grows normally on a host that carries an amber suppressor (e.g., HF4712) at 30° and 37°.

From the map positions of the two mutations one can easily predict that the mutation on the fragments A1, R6, and Z7 is the one involved in cell lysis and the mutation on A5, R7, Z3, and H7*a* is the one that causes a shortened A and A★ protein. To prove this, *am*6 was split up into its two single mutants. This was done by annealing fragment A1 or A5 to the *am*6 ssDNA (thereby covering only one of the mutations) and selecting for the single mutants among the progeny phages after spheroplast infection (see also Fig. 4). The mutation on A5, Z3, R7, and H7*a* can be picked up easily because it forms small plaques on C at 37° that do not plate on C at 30°; we call this one *am*62 (*A*). The other mutant (on A1, R6, and Z7) cannot be distinguished from *am*6 just by plating, but starting with the progeny phages after the spheroplast infection, only two growth cycles in C (nonsuppressor) are sufficient to have *am*6 outgrown by the single mutant that we call *am*61 (*E*).

Both single mutants were tested and their map positions and properties agreed with the expected results. *am*61 maps on A1, R6, and Z7, is lysis defective, and produces normal amounts of infective phages; *am*62 maps on A5, R7, Z3, and H7*a* and lyses its nonsuppressor host but makes phage particles that are mainly noninfective. The exact nature of this defect is not yet known.

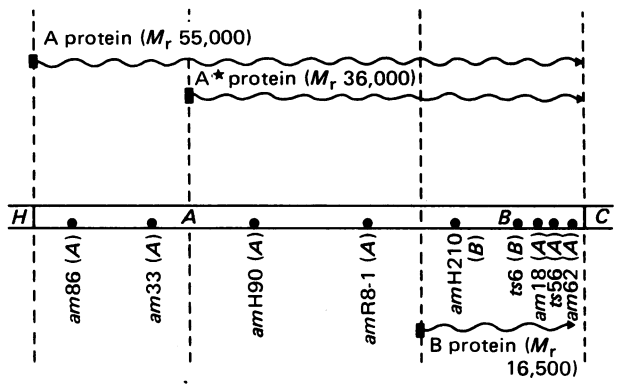

FIG. 6. Structure of the ϕX genome in the *A–B* region and the proteins it codes for. M_r, molecular weight.

The phage proteins that are specified for by these mutants are shown in Fig. 5. It is clear that *am62* still makes an A and A* protein of decreased size but that *am61* now has a normal A and A* protein. The wild-type phages that can be isolated from *am6* and *am62* by hybridization with A1 + A5 and A5, respectively, again have normal A and A* proteins. The way *am6* is split up and the properties of the resulting single mutants are schematized in Fig. 4.

We conclude from these experiments that the mutation that causes the shortened A and A* proteins is located on the fragments A5, R7, Z3, and H7*a* and that therefore *am62* is an amber mutant in the DNA sequence that codes for the A protein. *am62* maps between *ts116* (*B*) and *och6* (*C*) (Fig. 2), to the right side of all tested *B* mutants; this proves that the *A* gene overlaps the *B* gene.

DISCUSSION

The genetic organization of the *A–B* region, as it is deduced from our experiments, is given in Fig. 6. The gene that codes for the A and A* proteins does not terminate before the start of the *B* gene but its COOH-terminus is located close to the COOH-terminus of the *B* gene, thereby overlapping the *B* gene. The *B* gene is drawn in Fig. 6 entirely within the *A* gene. This is done because we have found *A* mutants on the right side of the R5/Z7*b* split, but no *B* mutants. Furthermore, from the amber protein fragment of *am16* (*B*) (9000 daltons) and the map position of *am16* (*B*), one can calculate that gene *B* ends at least before the H7*a*/H4 split, but it might well end much earlier. The end of the *A* gene is defined by (*i*) the presence of *A* mutants on fragment R7*b* [*ts56* (*A*), *am62* (*A*)], (*ii*) the size of the shortened A and A* proteins produced in *am62* infections, which shows that the *A* gene ends in R7*b* and runs 30 nucleotides at most into fragment H4, and (*iii*) the observation that in *am18* infections sometimes an extra protein band of 52,500 daltons is found which could well be the *am18* amber protein fragment. If this is correct then gene *A* has to end in fragment R7*b*. Together this proves that gene *A* ends quite close to gene *C* and that it runs at least over the greater part of gene *B*.

If gene *A* and gene *B* are translated in the same reading frame, then this would mean that a nonsense mutant in one of the two genes is automatically a nonsense mutant in the other. The existence of gene *A* and gene *B* as separate complementation groups immediately rules out this possibility. This is substantiated by the protein patterns of the amber mutants in this region: either the B protein or the A and A* proteins are missing. Also *B* mutants are not blocked in RF DNA replication, whereas *A* mutants are. Therefore, the two genes must be translated in different reading frames.

Whether the B protein can be synthesized on the same messenger as the A protein is unknown yet, but we know from the *in vitro* work of Smith and Sinsheimer (19) that there are initiation sites for transcription near the 5′ ends of gene *A* and gene *B*. This opens the possibility that the A and B proteins are made from different messengers.

How can such an overlap have come into existence? Recently Barrel *et al.* (3) have shown by DNA sequencing that gene *E* is a part of gene *D* and that both genes are read in different phases. They argue that it is hard to imagine that a series of single-base changes has created this situation. A change in a termination codon or a mutation that makes a new start signal seems a more likely way of creating such an overlap. In the case of the *A–B* overlap we think that the part of the A protein that is made in the overlap region is not too important for its function in DNA replication. When the end is removed [with *am62* (*A*), approximately 10 amino acids], the DNA replication seems not to be affected. It could therefore well be that, because of the removal of a stop codon, the *A* gene extended itself into gene *B*.

Recent DNA sequence results in Sanger's laboratory have also shown that gene *A* overlaps gene *B* and that gene *A* extends itself even beyond gene *B* (20). The presence of the two amber codons in *am6* has been confirmed. Its amber codon in gene *A* in fact shortens the A and A* proteins by six amino acids.

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