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Purification of the *gam* gene-product of bacteriophage Mu and determination of the nucleotide sequence of the *gam* gene

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ABSTRACT

The gam gene of bacteriophage Mu encodes a protein which protects linear double stranded DNA from exonuclease degradation in vitro and in vivo. We purified the Mu gam gene product to apparent homogeneity from cells in which it is over-produced from a plasmid clone. The purified protein is a dimer of identical subunits of 18.9 kd. It can aggregate DNA into large, rapidly sedimenting complexes and is a potent exonuclease inhibitor when bound to DNA. The N-terminal amino acid sequence of the purified protein was determined by automated degradation and the nucleotide sequence of the Mu gam gene is presented to accurately map its position in the Mu genome.

INTRODUCTION

Bacteriophage Mu is a temperate phage whose transmission involves non-homologous recombination with bacterial DNA and genetic transposition (1,2). The life-cycle of the phage is intiated by the injection of its genome from the phage coat into a susceptible prokaryotic host cell. This is followed by the integration of the Mu DNA into the host chromosome at a location which is essentially random. Phage integration requires the expression of the Mu-encoded transposase. Once integrated, the Mu DNA either exists as a stable quiescent prophage, a state maintained by the synthesis of a phage-coded repressor protein that blocks the expression of other viral functions, or the phage continues the production of tranposase and other factors involved in the transposition, thereby entering a lytic growth cycle where a cascade of replicative transposition events culminates in a burst of 50-100 phage particles approximately 60 minutes after injection.

The intimate link between Mu replication and transposition was first inferred from hybridization experiments which clearly showed that at various times after the induction of a lytic cycle in a lysogenic population of cells, the original prophage had not been excised from the host DNA, yet many copies of Mu could be found within the bacterial chromosome (3). More direct evidence of the replicative nature of Mu transposition came from the isolation of

"cointegrate structures" as transposition intermediates (1).

The Mu encoded transposase, the A gene-product, is absolutely required for Mu transposition, while the product of the Mu B gene amplifies the reaction (1). While the A and B gene products are the only Mu-encoded proteins necessary to carry out replicative transposition in vitro (4), the process is finely tuned by other phage proteins. Of particular interest are the proteins encoded by the "semi-essential early" (SEE) region of Mu. Mutants carrying deletions in this region or with polar upstream insertions form only "pin-point" plaques and may also have extended latent periods (5). A genetic analysis of this region has been hindered due to the semi-essential nature of the gene products, and a biochemical analysis of the purified proteins is an essential prerequisite to a complete understanding of the transposition reaction.

The SEE region of Mu spans 5 kb of DNA lying downstream of the Mu A and B genes but is transcribed from the same promoter as one large operon. The synthesis of at least 9 polypeptides, ranging in molecular weight (MW) between 7,000 and 22,000, is controlled by the SEE region, although the positions of the genes encoding them have not been mapped (6,7). A number of phenotypic effects have been traced to the region and putative "genes" have been proposed: The kil gene product is sufficient to kill the host cell even in the absence of Mu replication (8); the cim gene encodes a function that exerts a positive control on immunity (7,9); the arm gene product causes amplification of Mu replication (10); the lig (or topo) gene product causes partial complementation of bacterial and T4 mutants defective in ligase and topoisomerase (11,12); the gam gene product protects linear DNA from degradation by host exonucleases (13); and the expression of the sot protein in a host cell is necessary to obtain optimal transfection with naked linear Mu DNA extracted from phage particles by any of the standard methods (14).

The gam and sot phenotypes are both related to protection of DNA from exonuclease attack. Cloning experiments by Akroyd et al. (15) suggest that the gam and sot phenotypes are both manifestations of the same polypeptide of MW approximately 20,000 encoded by one small fragment of DNA of 1.6 kb. We have previously shown that the Mu gam gene product is a non-specific DNA binding protein that interacts with linear double-stranded DNA, preventing nucleolytic breakdown (15). In addition to stimulating transfection with linear Mu DNA, over-production of the gam and sot gene products in a recipient bacterium also stimulates the transformation frequency of a variety of linear plasmid DNA's (15). Delayed lysis and elevated lysogenization frequencies are exhibited by gam-deficient viruses, indicating the significance of pgam in phage development

and transposition (15).

In order to study the role of the gam gene-product in the Mu life-cycle, we have purified the protein (pgam) from hyper-producing cells. Its primary structure has been determined by DNA and protein sequencing methods and its biochemical properties have been partially characterized. The gam gene encodes a polypeptide of MW 18,900. The native gam protein is a dimer of identical subunits which binds to and protects duplex DNA from digestion by several exonucleases. It interacts with both single- and double-stranded DNAs, and it can aggregate DNA into large, rapidly sedimenting complexes.

MATERIALS AND METHODS

DNA sequencing. DNA sequencing was carried out using the dideoxy method developed by Sanger et al (16), using deoxyadenosine 5'-(α[^{35}S]thio) triphosphate as described by Biggin et al. (17). Sequencing was primed by the universal primer d(GTAAAACGACGGCCAGT).

The sequence of the amino-terminal 30 amino acids of the purified gam protein was derived on an Applied Biosystems 470A phase sequencer. PTH amino acids were identified by reverse phase high pressure liquid chromatography using a Perkin Elmer series 4 pump and CC85 B detector.

Enzyme assays DNA binding assays were carried out in 10 µl reaction mixtures containing 0.2 µg of Mu DNA, an aliquot of fraction to be assayed, 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 50 µg bovine serum albumin per ml, 1 mM $CaCl_2$. After incubation for 10 min at 37°C, 2 µl of 50% glycerol, 10mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.02% bromphenol blue were added and the samples were loaded onto 8 x 6 cm minigels composed of 1% agarose in TBE (42.3 g Tris Base, 22 g boric acid, and 3.7 g NaEDTA per L). Electrophoresis was at 125 V for 30 min, the gel was stained with ethidium bromide and photographed. DNA aggregation assays were carried out in 50 µl reactions containing 0.2 µg DNA in 25 mM Tris-HCl, pH 7.5, 15 mM KPO_4, 2 mM $MgCl_2$, 1 mM DTT, and 100 µg bovine serium albumin per ml. After incubation with varying amounts of pgam at 37°C for 15 min, the samples were spun in an Eppendorf centrifuge for 10 min. DNA in solution was measured by the Hoechst 33258 dye binding method of Labarca and Paigen (18).

Exonuclease III protection assays were carried out in 50 µl reactions containing 20 mM Tris-HCl, pH 7.9, 10 mM $MgSO_4$, 50 µg bovine serum albumin per ml, 10 mM ß-mercaptoethanol, and 0.2 µg ^{3}H-labeled Mu DNA. After a 10 min pre-incubation at 37°C in the presence of different levels of gam gene product, 10 units of exonuclease III (Bethesda Research Laboratories) were added and

incubation was continued at 37°C for 30 min. Samples were chilled on ice, and 20 µg of calf thymus DNA and 70 µl of 10% trichloracetic acid were added successively to each tube. Samples were mixed, incubated on ice for 10 min, and then centrifuged in an Eppendorf centrifuge at 4°C for 10 min. Acid soluble counts were determined by counting aliquots of the supernatant in a Triton X-100 based scintillation cocktail. We define a unit of pgam as the amount necessary to inhibit exonuclease III by 50% in the standard assay.

Purification of the gam protein (pgam). An overnight culture of strain MM 294 pJA 21 (15) was diluted 1:500 in 12 L of L-broth containing 100 µg/ml ampicilin and grown in a New Brunswick fermentor at 32°C to an OD_{600} of 1.0. Production of pgam was induced by shifting to 42°C and incubating for 90 min. Cells concentrated by ultrafiltration were centrifuged at 6,000 x g for 10 min and resuspended in 20 ml of 50 mM Tris-HCl, pH 8.0, 25% sucrose, and then frozen at -70°C until used for purification.

After thawing, the cells were diluted with an equal volume (approximately 28 ml) of lysis buffer containing 100 mM Tris-HCl, pH 7.5, 4 mM EDTA, 2 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonylfloride, 4 mg/ml lysozyme, and incubated 15 min on ice. The cells were redistributed into 60 ml Beckman polyallomar tubes and sonicated 4 times for 15 second intervals with one min cooling periods on ice between sonications. Ice cold polymin P neutralized to pH 7.0 was added to a final concentration of 0.5%, NaCl was added to a final concentration of 0.5M, and the solution was mixed at 0°C for 30 min. Precipitated DNA and associated proteins were pelleted by centrifugation for 10 min in a Beckman JA-21 rotor at 10,000 rpm. The gam protein remained in the supernatant. Solid ammonium sulfate was added to 60% saturation, the slurry was stirred at 0°C for 30 min, and then centrifuged at 12,000 x g for 30 min at 4°C. The pgam activity remained in the pellet which was resuspended in 20 ml of R Buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM DTT, and 0.1 mM EDTA), and dialysis was carried out for 3 h against R buffer plus 60 mM ammonium sulfate. The protein was passed over a mixed bed column containing 2.0 x 15 cm (50 ml) of hydroxyl apatite on the bottom and 2.0 x 15 cm (50 ml) of DEAE-cellulose on the top which was equilibrated with R buffer plus 60 mM ammonium sulfate. The gam protein passed through the column, was diluted 2-fold with R-buffer, and applied to a 2.0 x 31 cm (100 ml) DNA agarose column prepared as previously described (19). The column was washed with 300 ml of R buffer and then developed with a 400 ml linear 20 mM to 1 M NaCl gradient in R buffer. The gam protein eluted at approximately 300 mM NaCl and was concentrated by dialysis against 30% polyethylene glycol (MW 8000), 50 mM Tris-HCl, pH 7.6, 1

mM DTT. 5 ml of this preparation was applied to a 1.7 x 42 cm (100 ml) Ultrogel ACA 54 column equilibrated with R buffer plus 50 mM NaCl. The eluted protein was dialyzed against R buffer plus 50% glycerol and was stored at -20°C where it has remained stable for one year.

RESULTS

Isolation of pgam

Plasmid pJA21 (Fig. 1) which contains the Mu *gam* gene cloned on a 1.58 kb *EcoRI* fragment downstream from the λ promoter, controlled by the temperature sensitive λcI857 repressor, was used to produce high levels of the *gam* protein for purification. The construction of pJA21 has been described previously (15). The presence of pJA21 in thermo-induced *E. coli* cells greatly enhances the frequency with which Mu transfects these cells, and cell-extracts have been shown to contain a potent exonuclease inhibitor, indicating that the *gam* and *sot* gene products are expressed effectively by pJA21 (15). A protein of MW approximately 20,000 was found to be prominent in thermo-induced cells carrying pJA21 when analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE: Fig. 2 gel track B). Furthermore, a single polypeptide encoded by the Mu DNA cloned in pJA21 of MW 20,000 was identified in both mini- and maxi-cells carrying pJA21 after thermoinduction (Akroyd, thesis). There is, however, sufficient Mu DNA in the cloned fragment to encode

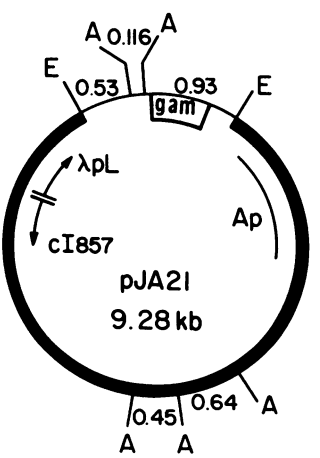

Fig. 1: Diagram of pJA21 showing the position of the *gam* gene relative to the *EcoRI* (E) and *AccI* (A) restriction endonuclease cleavage sites. Thin line, Mu DNA; thick line, plasmid vector DNA pXY228.

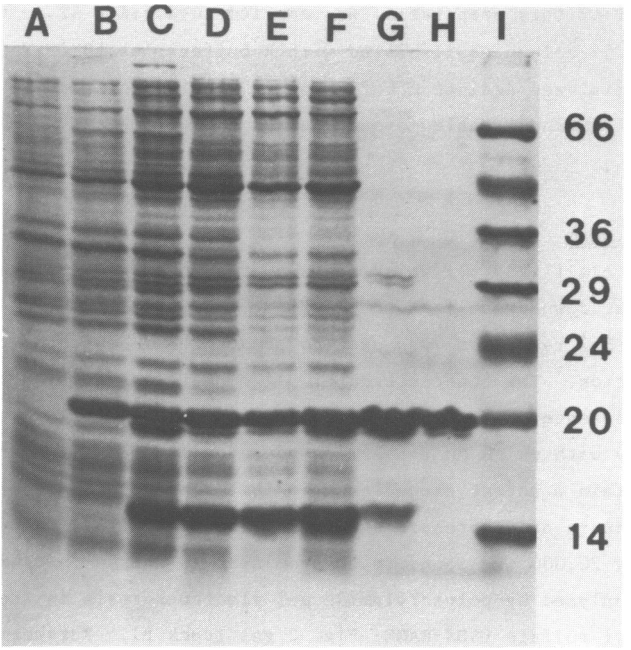

Fig. 2: Lysed whole cells carrying pJA21 both before and after thermoinduction were loaded in gel tracks A and B, respectively. Fractions I to VI of pgam purification (indicated in Table I) were loaded in tracks C to H, respectively. Molecular weight markers were loaded in gel track I and their sizes in kd are marked on the right hand side of the photograph.

more than one polypeptide and the possibility that another gene also lies on this fragment cannot be precluded at present.

A simple in vitro gam assay was developed based on previous observations that extracts from cells containing pJA21 conferred protection from recBC exonuclease on linear duplex DNA (15). Since the recBC exonuclease (exonuclease V) is difficult to obtain in a highly purified form, we used exonuclease III in our assay system, which degrades linear double-stranded DNA from the 3'-hydroxyl terminus and is commercially available in a highly purified form from a number of sources. Preliminary experiments indicated that exonuclease III was specifically inhibited by extracts of thermoinduced cells containing the pJA21 plasmid. Incubation of ^{3}H-Mu DNA with an extract having an overall protein concentration of 3µg/ml conferred 75% protection from exonuclease III digestion, while extracts of uninduced cells or induced plasmid-free cells showed no effect. Both SDS-PAGE (Fig. 2) and exonuclease III inhibition assays (Table 1) were employed throughout the course of enzyme isolation.

Table 1: Summary of exonuclease III inhibition assays on fractions I-VI from pgam purification.

Fraction	Total Volume (ml)	Total Protein (mg)	Total Units	Specific Activity
I. Cell lysate	60	--	--	--
II. Polymin P supernatant	56	--	--	--
III. Ammonium sulfate precipitate	20	2000	--	--
IV. Hydroxyl-apatite DEAE passthrough	62	1023	2.3×10^6	2.3×10^3
V. DNA-agarose	12	45	1×10^6	2×10^4
VI. Gel filtration	5	5.5	6×10^5	1×10^5

Since the pgam purification procedure exploits DNA binding properties of the protein, the first major step was to remove contaminating DNA from the crude thermo-induced extract of MM294 cells carrying pJA21 (Fraction I). This was achieved by a standard precipitation with polymin P to yield Fraction II (Table I). The residual polymin P remaining in solution after this step proved to be a potent exonuclease III inhibitor which was not removed by ammonium sulphate precipitation of the gam protein (Fraction III). A diagnostic feature of the polymin P inhibitor was its resistance to proteases; incubation of pgam with 50 µg proteinase K per ml abolished its effect, while polymin P inhibition was unaffected. In order to remove residual polymin P, as well as small nucleic acids, Fraction III was chromatographed through a mixed bed column composed of 50 ml hydroxylapatite poured on top of 50 ml DEAE-cellulose. The gam protein passed through this column, yielding Fraction IV which was immediately applied to a single-stranded DNA-agarose column and eluted in a salt gradient at about 0.3 M NaCl (Fraction V). Finally, after filtering through ultragel ACA54, 6 mg of highly purified gam protein were produced (Fraction VI; Fig. 2 lane H). A native protein eluting from the ACA54 column at the same position as chicken ovalbumin (MW 45,000) was identified as pgam due to its exonculease inhibition properties; this protein has a MW of approximately 20,000 on SDS-PAGE analysis, strongly suggesting that under native conditions pgam exists in solution as a dimer. Chromatography on ACA54

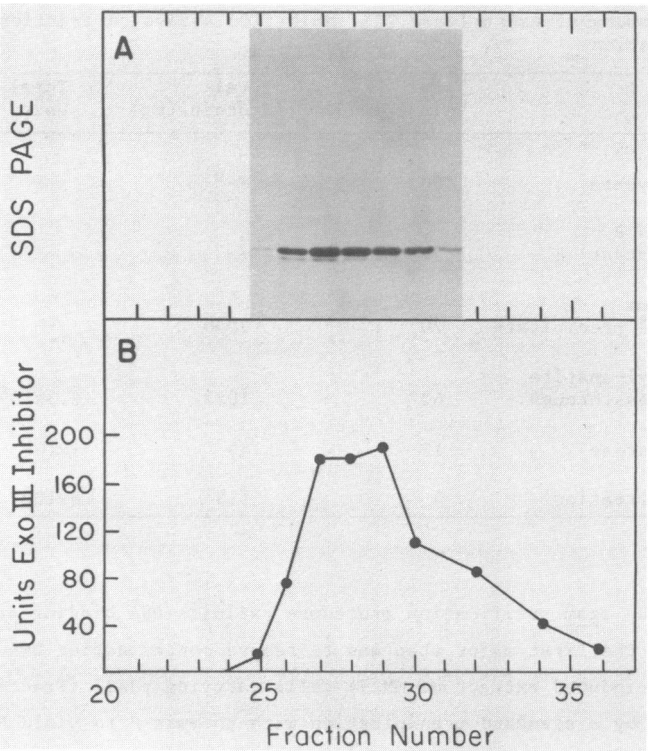

Fig. 3: Fractions from ACA54 gel filtration assayed A) by SDS-PAGE and B) for exonuclease III inhibition.

showed a clear coincidence between exonuclease inhibition and the 20,000 dalton protein which was produced abundantly in thermoinduced cells (Fig. 3).

On SDS-PAGE analysis, the purified protein exhibited a band running slightly ahead of the major staining band (Fig. 3a). After running long gels for 24 hours to achieve complete separation of the two bands, elution of top and bottom bands, and renaturation by the methods of Hager and Burgess (20), both bands were found to have identical biochemical properties indicating they both represented the gam gene-product.

Sequence of the gam gene and protein

The occurrence of two zones of pgam mobility in SDS-PAGE with apparently identical biochemical activity raised a question of the actual size of the protein monomer. After cloning into the M13 vector mp9, the coding region was sequenced by the dideoxy method (16,17). The gam gene was found to encode a polypeptide 174 amino acids long (Fig. 4). The purified protein was subjected to automated microsequencing, locating the first 30 amino acids at the amino

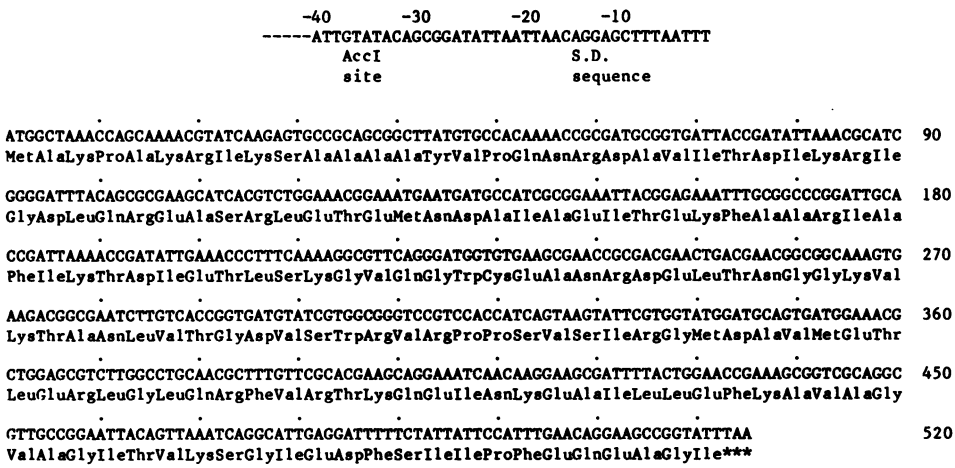

Fig. 4: Nucleotide sequence of the Mu gam gene given in the 5' to 3' direction from the AccI site located at 5.75 kb from the Mu left-hand end to the termination codon. The sequence of the coding (non-transcribed) strand is given. The numbers refer to the nucleotide position relative to the initiation codon, ATG. The Shine-Dalgano (S.D.) sequence is marked and the amino acid translation is given below the DNA sequence. This sequence enables the position of the structural gam gene to be pin-pointed accurately on the Mu genome.

terminal end and confirming the genetic assignment. The purified protein sequence corresponded to amino acid residues 2-31 in the genetic sequence. Thus, without the initiating methionine, the gam gene directs synthesis of a polypeptide chain with a MW of 18,900, which predicts a mobility closer to the faster moving of the two bands on SDS-PAGE (Fig. 3A). The reason for this unusual behavior on SDS-PAGE is unknown, but it may reflect protein modification or conformational flexibility in the polypeptide even in the presence of SDS.

DNA binding and aggregation assays

The binding of pgam to Mu DNA dramatically changed the electrophoretic mobility of the DNA in agarose gels (Fig. 5). At a critical ratio near 5 µg of protein per µg of Mu DNA, the Mu molecules did not migrate through agarose but remained in the gel pocket. The profile of this DNA binding assay performed on fractions from chromatography on ultragel ACA54 (Fig. 5) corresponded with both exonuclease III inhibition and the 20K protein observed on SDS-PAGE analysis (Fig. 3).

The dramatic change in gel mobility led us to analyze the sedimentation of pgam-DNA ensembles. pgam aggregated ^{3}H-labeled Mu DNA into complexes that

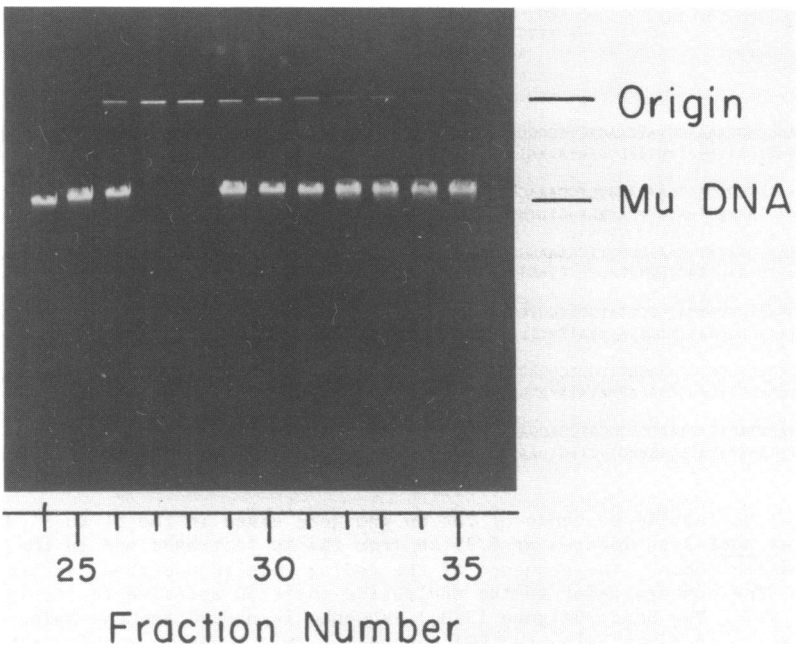

Fig. 5: Analysis of fractions from ACA54 chromatography using the DNA binding assay. DNA samples without the addition of any gam protein looked identical to the sample in the left-most gel track.

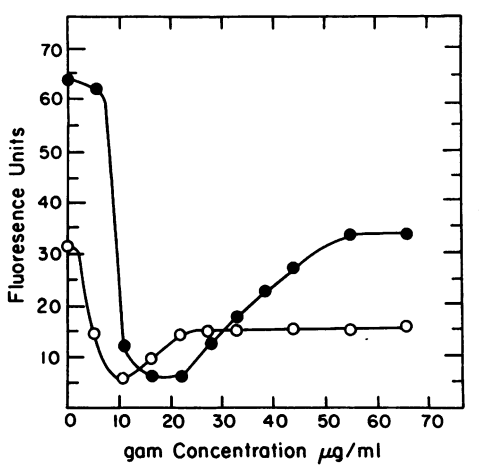

Fig. 6: The gam protein aggregates DNA. Incubation of pgam with supercoiled pUC18 plasmid DNA at concentrations of 6 μg DNA per ml (●) or 3 μg DNA per ml (○) was carried out at 37° for 15 min. Following Eppendorf centrifugation at room temperature for 10 min. 20 μl aliquots were removed and the DNA was measured by flourescence in the presence of Hoechst dye 33258 (18).

pelleted in 10 min of centrifugation at 12,000 x g (data not shown). This reaction was not unique to Mu sequences nor to linear DNA molecules. Supercoiled pSP65 plasmid (21) was maximally aggregated at a ratio of 1 pgam protein monomer per 6 bp of DNA; this critical ratio was observed at DNA concentrations of 3 µg/ml and 6 µg/ml (Fig. 6). Higher protein to DNA ratios approaching 1 pgam molecule per 3 bp of DNA caused some disaggregation. Protection of linear DNA from exoIII digestions occurred at pgam/DNA ratios that were 3-fold lower than those required to pellet DNA in an Eppendorf centrifuge Therefore, this type of aggregation is not necessary to block the nuclease action. Linear DNA also remained fully protected from exo-nuclease digestion at protein concentrations that caused slight disaggregation (data not shown). Since pgam is probably a dimer in solution, the aggregation properties suggest that the protein can crosslink DNA by binding to two molecules simultaneously.

DISCUSSION

DNA with unprotected ends is susceptible to exonucleolytic degradation. Bacterial viruses that generate linear DNA as intermediates in phage assembly or replication also produce proteins to protect their DNA from nuclease attack. Bacteriophge T7, T4, and λ each contain such proteins which are encoded by gene 1, gene 2, and gam respectively (22-24).

A combination of protein and DNA sequencing experiments prove that Mu gam is encoded by Mu DNA located between 5,801 and 6,322 bp from the left end (Fig. 4). Mu may encode more than one protein that protects phage DNA from degradation (27). Purified pgam protects linear DNA from degradation by exonuclease III (Fig. 3) and exonuclease V (data not shown) and cells which express this protein have elevated frequencies of transformation with linearized plasmid DNA (15). Therefore, pgam has the properties necessary and sufficient to explain the stabilization of linear DNA in Mu-infected cells. Besides its role in the phage life-cycle, the non-specific DNA binding activity of pgam and the stimulation of the frequency of transformation of a variety of linear DNA's in E. coli (15) is of particular interest, as it suggests a potential role for the gam protein as a "tool" in biological experiments in which linear intermediates are short-lived (e.g. in vitro and in vivo assays for recombination).

Williams and Radding (26) partially purified a protein of native MW between 40,000 and 80,000 from Mu-1 infected cells, which exhibited several properties in common with pgam purified from cells carrying pJA21, including

protection of linear DNA from digestion with exonuclease V and alteration of Mu DNA mobility in agarose gels. These authors stated that their partially purified inhibitor did not bind single stranded DNA or covalently closed supercoiled form I DNA. We observed single stranded DNA to be a competitor for pgam binding to linear duplex DNA (data not shown) and chromatography on a single-stranded DNA agarose column was one of the principal steps in pgam purification. Moreover, our preparation bound to and aggregated covalently closed supercoiled plasmid DNA. The reasons for these differences are not readily apparent. The monomer MW of the protein studied by Williams and Radding and the region of the Mu genome encoding it have not yet been determined to our knowledge. It is possible that the Mu gam protein behaves differently in the presence of modifying host proteins, or there could be subtle variations in the assays that explain the differences. In addition to pgam, another exonuclease III inhibitor was present in the extracts from cells carrying pJA21 and this protein, which correlated with the presence of a polypeptide of MW 25,000 in SDS-PAGE, resolved from pgam on single stranded DNA agarose chromatography. Sakaki et al. (28) also found a second recBC inhibitor when purifying the lambda gam protein. It is therefore possible that the proteins isolated by us and Williams and Radding (26) are not identical.

Agents that aggregate DNA can dramatically alter the end-products of DNA enzyme reactions. Condensation of substrates by long chain polymers, such as polyethylene glycol, greatly increases the reaction rates of DNA ligase reactions and promotes the formation of extremely long end products (30). DNA condensed with spermidine or other polyvalent cations is rapidly and efficiently catenated by topoisomerases into networks as large as the human chromosome (29). Condensed states are often associated with dramatic changes in DNA structure (31). The aggregation we observe with pgam is immediately reversed by protein denaturing agents like SDS and proteinase K treatment; it may involved physical crosslinking of DNA rather than the changes in DNA structure characteristic of polymer and charge neutralization aggregation.

How does the gam protein modulate the viral choice between lytic and lysogenic pathways of Mu development (15)? Current models for Mu transposition indicate that "simple" insertional transposition (akin to lysogeny) and replicative transposition (which is characteristic of viral lytic development) can both proceed from a single intermediate--a synaptic joint with the target sequence which is usually the host chromosome. Molecules with this property were predicted by Shapiro (32) and have been observed recently in vivo (33) and in vitro (34). If complete replication of the Shapiro intermediate proceeds

from one end of the transposon to the other, then replicative transposition results. Contrariwise, incomplete or interrupted replication results in "simple" insertional transposition events. If the synaptic junctions between Mu and the host chromosome bind pgam, the structure could be shielded from nucleases that normally resolve such structures (i.e., enzymes that resolve Holiday-junctions during homologous recombination). The net effect would be an increase in the frequency of complete replication. Definition of a precise role for this protein in transposition requires the isolation and genetic analysis of point mutations in gam together with an investigation of pgam activity in an in vitro transposition system which supports both replicative transposition and the formation of simple insertions as end products of the reaction (35).

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