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Purification of overexpressed *gam* gene protein from bacteriophage Mu by denaturation–renaturation techniques and a study of its DNA-binding properties

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Recombinant Mu *gam* gene protein (Mu GAM) synthesized in *Escherichia coli* accumulates in the form of insoluble inclusion bodies which, after cell lysis and low-speed centrifugation, can be recovered in the pellet fraction. This property was utilized in a purification procedure for Mu GAM based on guanidine hydrochloride denaturation–renaturation followed by a single DEAE-cellulose chromatographic step. The purified Mu GAM was shown by nitrocellulose-filter-binding experiments to bind with high affinity to linear double-stranded DNA and more weakly to supercoiled and single-stranded forms. Mu GAM protects linear DNA from degradation by a variety of exonucleases, but only weakly inhibits endonuclease activity. These results are in accord with a model of Mu GAM conferring protection from exonuclease activity by binding to the ends of DNA.

INTRODUCTION

The *gam* gene of the temperate phage Mu was so called when it was found that an early Mu gene could complement the *gam* gene of phage λ [1]. Interest in the Mu *gam* gene arose when preliminary experiments suggested that the Mu *gam* gene protein, GAM, acts in a different way from λ GAM. λ GAM has been shown to complex directly with exonuclease V, and to specifically inhibit the action of this enzyme [2,3].

The Mu *gam* gene had previously been cloned into an overexpression plasmid, pJA21, which enabled high-level synthesis of Mu GAM to be induced in *Escherichia coli* by a temperature shift [4,5]. Preliminary experiments using induced crude extracts of this strain indicated that Mu GAM inhibited exonuclease attack by interacting directly with DNA [4].

A small-scale purification of Mu GAM from induced cells using conventional protein purification techniques has been reported [6]. In the initial stages of the work reported here this method was found to give variable and low yields of Mu GAM. We observed that Mu GAM activity present in cell extracts was readily sedimented by low-speed centrifugation. Electron microscope investigation revealed that induced cells contained electron-dense micro-inclusions not present in uninduced cells. Although this property appeared to provide a useful purification step since approx. 90% of the Mu GAM was recovered in the pellet, attempts to resolubilize Mu GAM by conventional methods failed. However, active Mu GAM protein was solubilized by denaturation with guanidine hydrochloride (GdnHCl), and following a graded removal of the denaturant under optimal conditions for protein refolding, activity was regained. Previously this procedure has been applied successfully to other overproduced recombinant proteins expressed in *E. coli* [7–10]. Using these methods combined with a single chromatographic step Mu GAM was purified to homogeneity in high yield.

In this paper we report a reproducible purification procedure for overexpressed Mu GAM which gives a high yield of active homogeneous protein. With this purified protein we have been able to show that Mu GAM is a DNA-binding protein which inhibits a variety of exonucleases.

MATERIALS AND METHODS

Cultivation of bacteria

E. coli strain MM294 [*endA.recA supE, hsd R17*(r^-m^+)Tc^r] containing the plasmid pJA21 was used for this work. Plasmid pJA21 contains the *gam* gene cloned into the multicopy plasmid pXY228 downstream of the λpL promoter, from which expression is controlled by the temperature-sensitive repressor cI857 [5].

Cultures were grown in 2 litre baffled flasks containing 500 ml of L-broth supplemented with ampicillin (100 μg/ml). These were seeded with a 1% inoculum of an overnight culture grown in the same medium and then incubated at 32 °C in a shaking water bath, to an absorbance (A_{600}^{1cm}) of approx. 1.0.

The synthesis of Mu GAM was induced by rapidly increasing the temperature of the culture to 43 °C. This was achieved by adding 500 ml of L-broth at 62 °C to the culture and continuing the incubation at 43 °C for 90 min. Organisms were harvested by centrifugation at 10000 $g_{av.}$ for 5 min and resuspended in 8 ml of 50 mM-Tris/HCl buffer (pH 8.0) containing 25% sucrose. Samples were stored at −70 °C until used for purification.

Assay of Mu GAM activity

Mu GAM activity was measured by determining the extent to which Mu GAM protected DNA from degradation by exonuclease V, assessed by measuring radioactivity remaining in the supernatant after trichloroacetic acid precipitation of labelled DNA substrate. Duplicate reaction mixtures (50 μl) contained 67 mM-glycine/NaOH buffer (pH 9.4), 20 mM-$MgCl_2$, 8 mM-2-mercaptoethanol, 67 μg of bovine serum albumin/ml, 0.33 mM-ATP and 4 μg of ^{3}H-labelled Mu DNA/ml (13000 c.p.m.). After a 10 min preincubation at 35 °C in the presence of different concentrations of Mu GAM (0–20 μg/ml), an excess of exonuclease V (1 unit) was added. After gentle mixing, incubation was continued for 30 min, then the reaction was terminated by the addition of 20 μl of calf thymus DNA (1 mg/ml) and 70 μl of ice-cold trichloroacetic acid (10%, w/v). The acid-precipitated DNA was removed by centrifugation for 15 min at 4 °C in an

Abbreviations used: GdnHCl, guanidine hydrochloride; DTT, dithiothreitol.

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Eppendorf centrifuge and the supernatant (120 μl) was assayed for acid-soluble radioactivity in Optiphase MP scintillant (LKB).

Determination of protein concentration

The Bio-Rad protein assay, based on the dye-binding procedure of Bradford [11], was used to determine protein concentration.

Immunological studies

Antibodies were raised against Mu GAM in rabbits using standard procedures. Before injection, purified Mu GAM was subject to further purification by SDS/PAGE and recovered from the gel by electroelution. The antibodies were purified as described previously [12].

The Western immunoblotting technique was used to detect Mu GAM in protein samples after electrophoresis in SDS/polyacrylamide gels. The proteins were transferred to a nitrocellulose sheet using a semi-dry blotting technique [13] and were probed with a 1:1000 dilution of rabbit anti-GAM IgG using the Blotto system [14]. Horseradish-peroxidase-conjugated goat anti-(rabbit IgG) (Sigma) was used as a second antibody at a dilution of 1:1000 and blots were developed for 2 min with 0.4 mg of 3,3′-diaminobenzidine tetrahydrochloride/ml, 200 μl of $CoCl_2$ (10%, w/v) and 0.012% (v/v) H_2O_2.

Enzymes and isotopes

Exonuclease III, exonuclease VII, λ exonuclease and all of the restriction enzymes used in this work were obtained from Bethesda Research Laboratories, except exonuclease V which was obtained from Sigma. [*methyl*-^{3}H]Thymidine (40–60 Ci/mmol) was purchased from Amersham International.

Isolation of DNA and isotopic labelling

Mu DNA was isolated from phage after induction of an *E. coli* lysogen and labelled with [*methyl*-^{3}H]thymidine as described previously [15]. pBR322 DNA was isolated from a plasmid-containing *E. coli* strain by standard methods [16]. To radiolabel pBR322, [*methyl*-^{3}H]thymidine (5 μCi/ml) and uridine (400 mg/ml) were added to an exponential-phase culture of *E. coli* growing in a minimal salt and glucose medium supplemented with 0.2% casamino acids (M9CA medium [16]), immediately before plasmid amplification.

Nitrocellulose-filter-binding assay

Reaction mixtures (50 μl), containing binding buffer (10 mM-Tris/HCl (pH 8.0), 10 mM-$MgCl_2$, 10 mM-2-mercaptoethanol, 1 mM-EDTA and 0.5 mg of bovine serum albumin/ml) and 8 μg of [^{3}H]DNA/ml (2000 c.p.m.), were prepared in triplicate for each concentration of Mu GAM within the range 0–60 μg/ml and incubated for 30 min at 37 °C. Binding buffer minus bovine serum albumin (300 μl) was then added. The diluted reaction mixtures (340 μl) were filtered through alkali-treated nitrocellulose filters [17] (0.45 μm, diam. 9 mm, BA85) supported in microfilter assemblies (Schleicher and Schuell) and centrifuged for 1 min at 750 $g_{av.}$. The filters were then dried and the amount of [^{3}H]DNA retained on the filters was determined by scintillation counting using Optiphase MP scintillant (LKB).

Exonuclease inhibition assay

The inhibition of exonuclease V activity formed the basis of the assay used routinely for Mu GAM and is described above. The effect of Mu GAM on λ exonuclease activity was measured by a similar procedure. The reaction buffer contained 67 mM-glycine/KOH buffer (pH 9.4), 2.5 mM-$MgCl_2$ and 50 μg of bovine serum albumin/ml, conditions giving maximum λ exonuclease activity.

The inhibition of hydrolysis of double-stranded DNA by exonuclease III was measured essentially as described previously [6], except that the reaction mixture contained 50 mM-Tris/HCl buffer (pH 8.0), 5 mM-$MgCl_2$ and 10 mM-2-mercaptoethanol.

The substrate for exonuclease VII is single-stranded DNA, which was prepared by denaturation of linear ^{3}H-labelled pBR322 [18]. Inhibition of exonuclease VII activity was assayed by a similar procedure to that described above for the other exonucleases, except that the appropriate reaction buffer contained 67 mM-phosphate buffer (pH 7.9), 8.3 mM-EDTA and 10 mM-2-mercaptoethanol.

In all exonuclease inhibition assays the level of nuclease was saturating, i.e. for exonucleases III and V, 1 unit; λ exonuclease, 4 units; exonuclease VII, 2 units.

Endonuclease inhibition assay

Inhibition of restriction endonuclease activity was measured in reaction mixtures (50 μl) of 50 mM-Tris/HCl buffer (pH 8.0) containing 10 mM-$MgCl_2$ and 2 μg of linearized or supercoiled pBR322 DNA. After a 10 min preincubation in the presence of different concentrations of Mu GAM (0–100 μg/ml), 10 units of restriction endonuclease was added and the incubation was continued at 37 °C for 1 h. The samples were then extracted with phenol and 2 μl of loading buffer [40% (w/v) sucrose/0.25% Bromophenol Blue] was added to 8 μl of the deproteinated reaction mixture before analysis by gel electrophoresis. Agarose gels (0.8%, w/v) in 100 mM-Tris base, 100 mM-boric acid, 20 mM-EDTA and 0.05% ethidium bromide were run for approx. 3 h at 50 V and then photographed over u.v. illumination with Polaroid film (type 667) in order to detect changes in the expected restriction pattern.

RESULTS

Induction and localization of Mu GAM

Samples from a culture of the *E. coli* strain MM294/pJA21 were taken before and after induction of Mu GAM synthesis, as described in the Materials and methods section, and examined by transmission electron microscopy. Fig. 1 shows the presence of amorphous electron-dense aggregates in induced cells which are not present in uninduced cells. The formation of such aggregates as a result of over-production has been reported for the protein products of several cloned prokaryotic and eukaryotic genes. Such aggregates or inclusion bodies are readily sedimented by low-speed centrifugation (5000–12000 $g_{av.}$) [7–10].

Fig. 2(*a*) shows that extracts from the induced cells, when analysed by SDS/PAGE, contained high levels of a polypeptide of M_r 20000, which corresponds to the M_r of the *gam* gene protein (lane c). A Western blot using antisera raised against purified active Mu GAM confirmed this designation (Fig. 2*b*).

After low-speed centrifugation of the crude extract the majority of the Mu GAM was found to be associated with the pellet fraction (Fig. 2*a*, lane e) rather than in the supernatant (Fig. 2*a*, lane d). These data indicate that the overproduction of Mu GAM in *E. coli* results in the formation of an aggregated form of Mu GAM, which is readily sedimented by low-speed centrifugation.

Mu GAM in the pelleted material was not solubilized by increasing the salt concentration (up to 4 M-KCl) or by altering the pH (within the range 7.5–9). It has been found for other proteins that the chaotropic agent GdnHCl will effectively resolubilize aggregates of this type by denaturation. Judicious selection of renaturing conditions allows the polypeptide to refold, and biological activity is recovered [7,8]. When this type of methodology was applied to the pellet containing Mu GAM, a successful purification procedure was developed as described below.

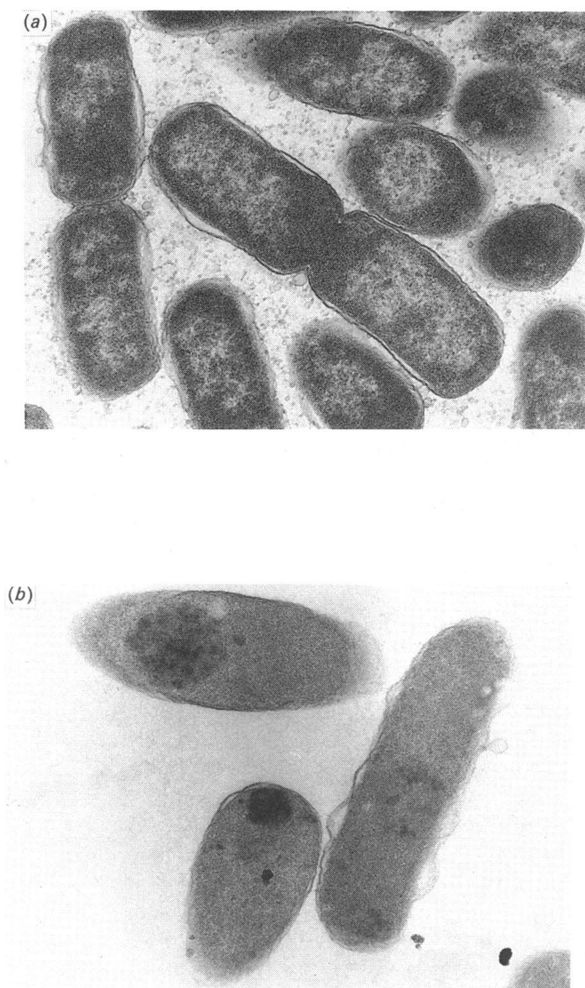

Fig. 1. Electron micrographs of *E. coli* (strain MM294/pJA21) before and after induction of Mu GAM synthesis

Before and after induction of cultures, samples (20 ml) were taken and centrifuged for 5 min at 10000 $g_{av.}$. The resulting pellet of whole cells was fixed first in 5% glutaraldehyde and then in 1% OsO_4, and prestained with 0.5% uranyl acetate. After dehydration through a series of alcohols, the cells were embedded in Sperr's resin. Sections were cut using a LKB Ultrotome III ultramicrotome, stained in lead citrate and examined on a JEOL 100C electron microscope at 80 kV. (*a*) Transmission electron micrograph of bacterial cells before induction of Mu GAM synthesis and (*b*) after induction. Magnification ×19200.

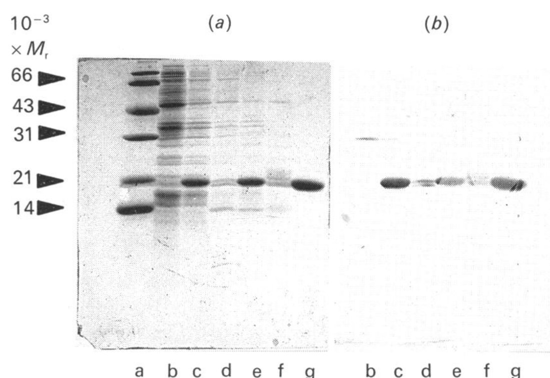

Fig. 2. Purification of Mu GAM from *E. coli*

(*a*) Gel electrophoresis was performed in a 15% (w/v) acrylamide gel under conditions described by Weber & Osborn [19] and stained with Coomassie Blue R250. Lane a, M_r markers (Bio-Rad) as indicated on the left of the photograph; lanes b and c, total cell proteins from MM294/pJA21 before and after induction respectively; lane d, supernatant after lysis and centrifugation; lane e, pellet after lysis and centrifugation; lane f, flow-through from DEAE-cellulose chromatography; lane g, after refolding. In lanes c–g, 5 μg of total protein was loaded on the gel. (*b*) Western blot of gel run under similar conditions to that in (*a*) and then treated as described in the Materials and methods section. Lanes b–g are the same as lanes b–g in (*a*).

Purification of Mu GAM

Step 1: preparation of the crude extract. After thawing, the cells (4.4 g wet weight) were diluted with an equal volume (8 ml) of lysis buffer containing 100 mM-Tris/HCl buffer (pH 7.5), 4 mM-EDTA, 2 mM-dithiothreitol (DTT), 0.4 mM-phenylmethane-sulphonyl fluoride and 4 mg of lysozyme/ml, and incubated on ice for 15 min. The suspension was sonicated on ice for six 15 s intervals at an amplitude of 12, with 1 min cooling periods, using an MSE sonicator.

The lysate was centrifuged (10000 $g_{av.}$) for 10 min at 4 °C and the supernatant was discarded. The pellet was then washed in lysis buffer without lysozyme and centrifuged (10000 $g_{av.}$) for 30 min at 4 °C.

Step 2: solubilization of the pellet protein. The washed pellet was resuspended in 5 ml of buffer A (50 mM-phosphate, 1 mM-EDTA, 15 mM-2-mercaptoethanol, 7 M-GdnHCl, pH 7.0). The suspension was stirred at 4 °C overnight and clarified by centrifuging at 25000 $g_{av.}$ for 3 h at 4 °C. The pellet was discarded and the supernatant was dialysed for a total of 20 h against four changes of 250 ml of buffer B (15 mM-Tris/HCl, 15 mM-2-mercaptoethanol, 75 mM-NaCl, 8 M-urea, pH 8.3).

Step 3: chromatography on DEAE-cellulose. The supernatant containing Mu GAM was loaded on a 6 cm × 1.5 cm column of DEAE-cellulose equilibrated with buffer B. The column was developed by a downward flow of 20 ml·h^{-1} at room temperature. Mu GAM was not bound to the column and appeared in the flow-through.

Step 4: renaturation. The flow-through fraction containing Mu GAM was adjusted to pH 10 with NaOH (1 M) and dialysed for a total of 24 h against three changes (1 litre) of buffer C (14 mM-Tris/HCl, 0.1% 2-mercaptoethanol, 1 M urea, pH 7.0), followed by dialysis for a total of 24 h against three changes (1 litre) of 10 mM-Tris/HCl buffer (pH 7.5) containing 0.5 mM-DTT and 0.05 mM-EDTA. Some precipitation occurred during this procedure. The precipitate was removed by centrifugation (25000 $g_{av.}$) for 30 min. The protein yield at this stage was 22 ml at 117 μg/ml. This protein was concentrated by freeze-drying and the dried material was resuspended in water (3 ml). A small amount of material did not redissolve and was removed by centrifugation for 15 min in an Eppendorf centrifuge.

The yield from 1 litre of induced cells was 2 mg of Mu GAM. The purified protein migrated as a single band when subjected to SDS/PAGE (Fig. 2*a*, lane g). Comparison of this lane with lane f (Fig. 2*a*), the flow-through material obtained in step 3, shows that removal of the insoluble precipitate which formed during renaturation resulted in a significant purification of resolubilized Mu GAM. Mu GAM purified by this method produced 90% inhibition when 2 μg of the purified protein/ml was added to the standard assay for Mu GAM activity described in the Materials and methods section (Fig. 3).

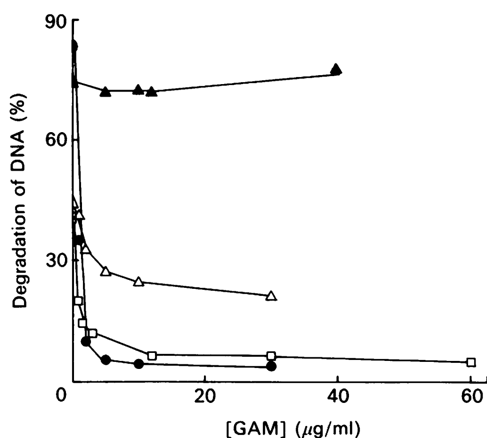

Fig. 3. Effect of Mu GAM concentration on the activities of exonuclease III, exonuclease V, exonuclease VII and λ exonuclease

Using the assay as described in the Materials and methods section, the degradation of [^{3}H]DNA (2 μg/ml) by exonuclease III (□), of [^{3}H]DNA (4 μg/ml, 13000 c.p.m.) by exonuclease V (●) and λ exonuclease (△) and of pBR322 [^{3}H]DNA (single-stranded linear, 4 μg/ml, 2000 c.p.m.) by exonuclease VII (▲) was measured at increasing concentrations of Mu GAM (0–60 μg/ml) in duplicate. The degradation, representing the number of acid-soluble counts as a percentage of the total counts in the reaction mixture, is plotted as a function of the Mu GAM concentrations indicated.

Table 1. Summary of the substrate specificities of the exonucleases investigated and their sensitivities to inhibition by Mu GAM

Enzyme	Exonuclease substrate characteristics	Inhibition by: Mu GAM	λ GAM*
Exonuclease III	Double-strand specific Hydrolyses in 3′ → 5′ direction only	Yes	No
Exonuclease V	ATP-dependent; hydrolyses in both 3′ → 5′ and 5′ → 3′ directions		
	Double-strand exonuclease activity	Yes	Yes
	Single-strand exonuclease activity†	No	Yes
Exonuclease VII	Single-strand specific Hydrolyses in both 3′ → 5′ and 5′ → 3′ directions	No	No
λ Exonuclease	Double-strand specific Hydrolyses in 5′ → 3′ direction only	Yes	No

* Ref. [3].
† The rate of degradation of single-stranded DNA is always less than that of duplex DNA.

Inhibition of deoxyribonuclease activity by Mu GAM

Exonucleases. The effect of the purified Mu GAM on the activity of four exonucleases with different substrate specificities (Table 1) was then studied.

Fig. 3 shows that purified Mu GAM inhibited the activities of the double-stranded DNA-specific nucleases (exonuclease III, exonuclease V and λ exonuclease) with Mu DNA as a substrate. Mu GAM did not inhibit λ exonuclease to the same extent as exonucleases III and V. This could be due to λ exonuclease binding more tightly to DNA than exonucleases III and V, and thus Mu GAM cannot compete as effectively with the λ exonuclease for binding sites on the DNA. Under similar conditions, Mu GAM did not inhibit exonuclease VII (specific for single-stranded DNA) from degrading denatured pBR322 DNA.

Table 2. Effects of different types of DNA ends on the ability of Mu GAM to inhibit exonucleases

The exonuclease (Exo) inhibition assay was carried out as described in the Materials and methods section. The DNA concentration was 4 μg/ml and the Mu GAM concentration 20 μg/ml.

	Degradation (%) by:			
	ExoIII		ExoV	
	−GAM	+GAM	−GAM	+GAM
Mu DNA	35	17	83	17
pBR322 linearized with *Sph*I (3′ 'sticky'-ended)	20	12	84	15
pBR322 linearized with *Bam*HI (5′ 'sticky'-ended)	42	13	68	14
pBR322 linearized with *Eco*RV (blunt ended)	62	17	93	30

These results are summarized in Table 1 together with a comparison of the available data for λ GAM [3]. The range of exonuclease activities inhibited by Mu GAM and λ GAM is clearly different, since λ GAM only inhibits exonuclease V. In the case of λ GAM, it has been established that specific inhibition occurs by λ GAM binding to the exonuclease [3]. Mu GAM, however, inhibited all three double-strand-specific exonucleases which were tested, suggesting that the mode of action of Mu GAM is different from that of λ GAM.

The inhibition of exonuclease activity by purified Mu GAM was not limited to Mu DNA as a substrate, since the degradation of linearized pBR322 by exonucleases III and V was also inhibited (Table 2). These data also show that Mu GAM can confer protection from hydrolysis on linear double-stranded DNA with either flush or 'sticky' ends.

Restriction endonucleases. The inhibitory effect of Mu GAM on endonucleases was next investigated. Restriction endonucleases which require NaCl for their activity (e.g. *Eco*RI) could not be tested in this system, as it was found that at the concentrations used in standard restriction enzyme assays the NaCl itself inhibited Mu GAM activity (results not shown). We therefore looked at the inhibitory effect of Mu GAM on three restriction endonucleases, *Rsa*I, *Cla*I and *Acc*I, none of which require NaCl in their reaction mixtures.

The results obtained with all three of these endonucleases were similar. Fig. 4 shows the results obtained with *Rsa*I. It can be seen that over a wide range of Mu GAM concentrations the intensities of the four primary *Rsa*I fragments, derived from linearized pBR322 DNA as a substrate, did not change significantly. This indicates that Mu GAM does not strongly inhibit *Rsa*I activity. However, partial digestion fragments were seen, particularly at high Mu GAM concentrations, but always as minor products.

The size distribution of the partial digestion fragments is consistent with all three sites being protected by Mu GAM to the same extent. At lower Mu GAM concentrations, the larger fragments are more readily detectable because they stain more intensely with ethidium bromide. These results are consistent

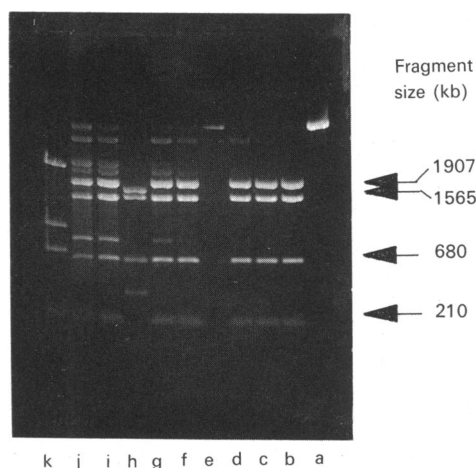

Fig. 4. Effect of Mu GAM concentration on the activity of the restriction endonuclease *Rsa*I

Reaction mixtures (50 μl) containing 40 μg of pBR322/ml (linearized with *Bam*HI), *Rsa*I (10 units) and various concentrations of Mu GAM were treated as described in the Materials and methods section. After agarose gel electrophoresis in the presence of 0.05% (w/v) ethidium bromide, the DNA fragments were visualized by u.v. illumination before photography. Lane a, linearized DNA; lane b, no Mu GAM was added. Lanes c, d, f, g and i contained increasing concentrations of Mu GAM of 10, 20, 25, 50 and 100 μg/ml respectively. Lane j contained only 20 μg of DNA/ml, but 100 μg of Mu GAM/ml. The M_r markers used were: lane e, plasmid pACYC184, 4.0 kb; lane h, pBR322/*Sph*I and cut with *Rsa*I, fragment sizes 401, 680, 1565, 1716; lane k, pBR322/*Eco*RV and cut with *Bgl*I, fragment sizes 234, 747, 1063, 2319.

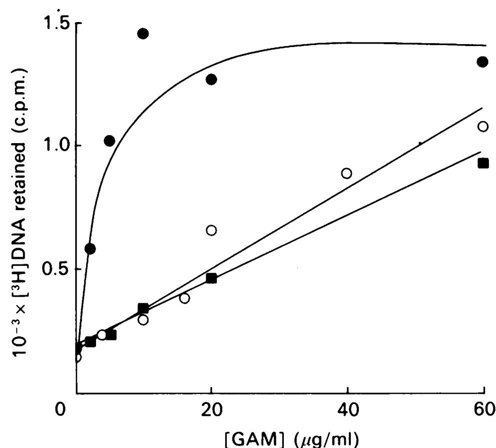

Fig. 5. Comparison of the retention of double-stranded linear, supercoiled and single-strand linear DNA complexed with Mu GAM on nitrocellulose filters

pBR322 [^{3}H]DNA (8 μg/ml, 2000 c.p.m.), double-stranded linear (●), supercoiled (■) or single-strand linear (○) were incubated in binding buffer (50 μl) with increasing amounts of Mu GAM from 0–60 μg/ml for 30 min at 37 °C. The reaction mixtures were made up in triplicate for each Mu GAM concentration tested. The amount of radioactivity retained on the filter, measured as described in the Materials and methods section, is plotted as a function of Mu GAM concentration.

with weak non-specific binding of Mu GAM to central regions of DNA.

Nitrocellulose-filter-binding studies

The results reported so far strongly suggest that Mu GAM protects DNA from exonuclease attack by binding directly to the DNA. However, all of the evidence is indirect. In order to get direct confirmation of this point, a series of nitrocellulose-filter-binding experiments were carried out. This technique has become a standard method for detecting the DNA-binding activity of proteins [20]. It is based on the property of nitrocellulose to retain protein–DNA complexes under conditions where free nucleic acid is not bound. Fig. 5 shows that linear double-stranded DNA is retained by nitrocellulose in the presence of Mu GAM. As the amount of Mu GAM was increased relative to DNA, typical saturation kinetics were observed, with saturation occurring at a weight ratio of Mu GAM to DNA of 5. The affinity of Mu GAM for linear and supercoiled DNA was also measured. From Fig. 5, it can be seen that for a given DNA concentration (8 μg/ml) more linear DNA than supercoiled DNA was retained at each Mu GAM concentration tested. However, with supercoiled DNA, typical saturation kinetics were not observed; instead, a straight-line relationship was obtained, and saturation was not observed even at the highest concentration of Mu GAM tested (60 μg/ml). This behaviour is typical of non-specific protein–DNA interactions [21]. These results provide direct evidence for the binding of Mu GAM to DNA. They also show that Mu GAM can bind to linear duplex, single-stranded and supercoiled DNA, but has the highest affinity for linear duplex DNA.

DISCUSSION

The purification procedure described here using denaturation–renaturation techniques is more reproducible in our hands than that described previously using conventional techniques [6]. A 4-fold higher yield of Mu GAM, 20-fold more effective in inhibiting exonuclease III (μg of protein required to produce a 50% inhibition of nuclease activity) was obtained. This higher activity indicates that the conditions used for renaturation have allowed effective refolding of the denatured Mu GAM and that the renatured product is not significantly contaminated with inactive protein.

The nitrocellulose-filter-binding data reported above show clearly that purified Mu GAM binds to DNA; with high affinity to the linear duplex form and more weakly to covalently closed circular and single-stranded DNA. The characteristics of the binding are not affected by the source of the DNA employed in the test. With linear duplex DNA (having flush or 'sticky' ends), Mu GAM affords efficient protection against degradation by a variety of exonucleases with different specificities. Some protection against endonucleases is also observed, but at much lower efficiency. These inhibition data, together with the nitrocellulose-filter-binding studies discussed above, are consistent with Mu GAM binding to the ends of double-stranded DNA.

Do these properties of Mu GAM suggest any role for the protein in the Mu life-cycle? It has been reported that many linear double-stranded DNA phages, including λ, T2, T3, T4, T5, T6, T7, P1 and P22 [22–24], synthesize a product during lytic growth that inhibits exonuclease activity. Two types of explanation have been proffered for this widespread activity. One is that exonuclease V inhibits rolling circle replication, often used by phages to generate concatemers; the other that linear phage genomes are susceptible to exonuclease attack, in particular immediately after injection. In the case of phage λ, rolling circle replication seems to be the sensitive stage with regard to exonuclease V, and the λ *gam* gene synthesizes a protein which binds directly to the exonuclease, so affording protection [2,3]. The only other case where there is persuasive experimental evidence for involvement of a DNA-binding protein is with phage T4, where the product of gene 2 appears to have a dual role. It binds to the termini of DNA molecules, so protecting

injected virion DNA from degradation, and also has a morphogenetic function in the proper assembly of T4 DNA within the phage head [25].

Phages of Mu contain linear double-stranded DNA, so the injected virion DNA requires protection against exonuclease attack. In terms of its affinity to DNA, the simplest role for Mu GAM in the Mu life cycle would be that of binding to the ends of virion Mu DNA and so affording protection. However, it is known that this function is carried out by the product of the N gene of Mu, a 64000-M_r protein compared with the 20000 M_r of Mu GAM [26]. Moreover, because mutants in the N gene are non-viable, Mu GAM cannot substitute for the N protein. Apparently, as with the T4 gene 2 protein, the N gene product has an additional function to that of inhibiting exonuclease attack, and this could relate to the bringing together of the ends of the injected Mu DNA to form the non-covalently bound supercoiled circles which are the precursor for Mu integration [27].

It seems then that whatever role Mu GAM has in the Mu cycle, it is not to do with protecting virion DNA from exonucleases. Unlike N, a functional *gam* gene is not essential for Mu growth, as there are viable Mu mutants which contain deletions that cover part of the *gam* gene [28]. A GAM-like protein could nonetheless be involved in some aspect of phage growth, but provided if necessary by a cellular gene. The next most likely stage at which Mu GAM (or its equivalent) could act is replication. Here the target would be the act of transposition rather than rolling circle replication, as Mu is known to replicate its genome by successive rounds of transposition. Studies *in vitro* with mini-Mu derivatives have identified an intermediate structure in the complete strand-transfer reaction which comprises the finished act of transposition. This intermediate is formed when a supercoiled mini-Mu donor plasmid is incubated with two DNA-binding proteins. One of these is the site-specific Mu transposase coded by the MuA gene, and the other, HU, is the non-specific histone-like protein encoded by *E. coli* [29]. Furthermore, later experiments have shown that the presence of another host-encoded DNA-binding protein, IHF, which has both specific and non-specific binding attributes with regard to the donor mini-Mu, can reduce the requirement for superhelicity in the donor DNA and so affect the level of transposition [30]. It remains a distinct possibility, therefore, that in the more complex situation *in vivo* an extra degree of modulation in transposition activity could come about from the general DNA-binding properties of Mu GAM.

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