Effects of purified SeqA protein on oriC-dependent DNA replication in vitro

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In vivo studies suggest that the Escherichia coli SeqA protein modulates replication initiation in two ways: by delaying initiation and by sequestering newly replicated origins from undergoing re-replication. As a first approach towards understanding the biochemical bases for these effects, we have examined the effects of purified SeqA protein on replication reactions performed in vitro on an oriC plasmid. Our results demonstrate that SeqA directly affects the biochemical events occurring at oriC. First, SeqA inhibits formation of the pre-priming complex. Secondly, SeqA can inhibit replication from an established pre-priming complex, without disrupting the complex. Thirdly, SeqA alters the dependence of the replication system on DnaA protein concentration, stimulating replication at low concentrations of DnaA. Our data suggest that SeqA participates in the assembly of initiation-competent complexes at oriC and, at a later stage, influences the behaviour of these complexes.

Keywords: DnaA/Escherichia coli/initiation of DNA replication/in vitro replication/SeqA

Introduction

Replication of the Escherichia coli chromosome starts at a single locus, oriC. Initiation of replication is precisely controlled in several aspects. During steady-state growth, initiation occurs at a specific time in the cell division cycle and at a specific cell mass. Normally this coupling of initiation to cell growth is extremely tight; the coefficient of variation in individual cell mass at the time of initiation is <9% (Boye et al., 1996). In rapidly growing cells containing several copies of oriC, all origins are initiated in synchrony (Skarstad et al., 1986). Furthermore, a mechanism called sequestration acts at oriC to ensure that a newly replicated origin is temporarily refractory to initiation (Russell and Zinder, 1987; Ogden et al., 1988; Campbell and Kleckner, 1990; Landoulsi et al., 1990; Lu et al., 1994).

Genetic and physiological studies suggest that SeqA plays important roles in control of replication (Lu et al., 1994; von Freiesleben et al., 1994; Boye et al., 1996). First, SeqA is a negative regulator of the onset of DNA replication. This role was inferred originally from the effects of SeqA elimination or SeqA overproduction in mutant strains compromised for initiation. Subsequent studies specifically identified SeqA as a regulatory factor for initiation. In the absence of SeqA, initiation occurs at a lower cell mass compared with wild-type cells and origins may be initiated twice in the same cell division period (Boye et al., 1996). Secondly, SeqA is essential for sequestration (Lu et al., 1994). These two effects of SeqA are distinguished in part by the methylation status of GATC sites in oriC: initiation occurs on fully methylated DNA whereas sequestration affects oriC in the hemimethylated state that arises as a result of its replication.

Biochemical analysis has shown that SeqA binds oriC in a methylation-modulated fashion (Slater et al., 1995). SeqA binds fully methylated oriC specifically via a highly co-operative but relatively low affinity interaction. This binding requires multiple determinants throughout oriC (Slater et al., 1995), probably explaining why SeqA is reported not to bind to a fully methylated 70 bp oriC fragment (Brendler et al., 1995). SeqA also binds hemimethylated oriC and certain other hemimethylated DNAs by a higher affinity, less co-operative interaction (Brendler et al., 1995; Slater et al., 1995). Unmethylated oriC is not bound by SeqA (Slater et al., 1995).

There is no information regarding the precise biochemical stage(s) of initiation at which SeqA exerts its effect(s). Genetic studies seem to implicate open complex formation as a critical step because the mutants affected by SeqA elimination or overproduction appear to be specifically defective in that stage; furthermore, manipulation of SeqA levels has no effect on a mutant whose corresponding biochemical defect is just after open complex formation (Lu et al., 1994). Physiological studies provide no additional information as to the specific point of SeqA action: the time of replication initiation is inferred from flow cytometry analysis, which essentially monitors the cell mass at which bulk synthesis of new DNA begins. Thus, an effect of SeqA on initiation timing could be executed at any point at or before escape of the replication fork from the origin region.

Here we present a biochemical investigation of the effects of purified SeqA protein on replication initiation from oriC in vitro. These studies provide direct biochemical evidence for a functional interaction between SeqA and DnaA proteins at oriC during early stages of the initiation process. We demonstrate that SeqA can act negatively both on the formation of the pre-priming complex and on replication from this complex once formed, and suggest that SeqA ensures development of a fully elaborated nucleoprotein complex at oriC.

Results

Effects of SeqA protein in reconstituted replication reactions

Replication of oriC plasmids in vitro can be reconstituted with purified replication proteins (Kaguni and Kornberg,
SeqA at 10 nM corresponds to three SeqA molecules per reaction. DNA replication is measured in nucleotides (nt) synthesized as described in Materials and methods (RNA polymerase-independent mixture on ice before incubation at 29°C). The replication reaction was indicated amounts of SeqA or SeqA buffer were added to the reaction buffer (Figure 1). When added before shifting to 29°C, which permits open complex formation. Total nucleotide incorporation was assayed after 20 min. Under these conditions, the presence of SeqA significantly inhibited replication, over and above a small amount of inhibition due to the presence of salt in the SeqA storage buffer (Figure 1). When added before shifting to 29°C, the order of SeqA addition relative to the other replication proteins did not affect the inhibition. Also, the same level of inhibition was observed if SeqA was allowed to bind to the DNA template [by pre-incubating SeqA and template (pBSoriC) at 29 or 37°C for 20–25 min] before adding the other proteins, or if SeqA was pre-incubated with the complete set of replication proteins (at 0°C) prior to addition of pBSoriC (data not shown).

In vivo, the initial DNA duplex opening reaction at oriC is dependent on transcriptional activity by RNA polymerase. In the in vitro replication system used above, initiation is independent of transcription. This difference arises because the level of free, unrestrained supercoils in the purified plasmid DNA template is higher than in a native bacterial chromosome (Ogawa et al., 1985; van der Ende et al., 1985), since supercoils are restrained in vivo by the binding of structure-modifying proteins (Pettijohn, 1988; Drlica, 1992). Dependence on transcriptional activation can be achieved in vitro by reducing the level of free supercoils in the template DNA to the in vivo level by adding a high level of HU protein (Baker and Kornberg, 1988; Skarstad et al., 1990). SeqA inhibited both RNA polymerase-dependent and -independent replication reactions to a similar extent (data not shown).

The ability of SeqA to inhibit DNA replication was sensitive to the methylation status of the template plasmid: at low SeqA levels (~150 nM), inhibition was observed on a fully methylated template but not on an unmethylated template (Figure 2). This is parallel to what is observed for SeqA in binding oriC-containing DNA fragments in gel retardation assays (Slater et al., 1995). A further inhibition of the reaction was observed at higher SeqA concentrations, and this inhibition appeared to be independent of methylation status. This effect may be analogous to the in vivo finding that overproduction of SeqA protein is inhibitory to growth of a dam strain (Lu et al., 1994).

In the standard reconstituted replication reaction, the DnaA concentration used (~50 nM) is that required to give maximal DNA synthesis. In trying to make the reaction more sensitive to SeqA, we carried out reconstituted reactions at other DnaA concentrations. We found that at low DnaA concentrations (~20 nM), SeqA (at 180 nM, sufficient to give a significant inhibition at normal amounts of DnaA) actually stimulated the reaction considerably (Figure 3). This stimulatory effect has also been confirmed in the RNA polymerase-dependent replication reaction. Furthermore, stimulation was observed irrespective of the order of addition of DnaA and SeqA into the reaction (data not shown).

**Effects of SeqA on formation of pre-priming complexes**

When supercoiled oriC plasmid, ATP, DnaA, DnaB, DnaC and HU or IHF proteins are incubated together at an appropriate temperature, the pre-priming complex forms: DnaA protein, assisted by HU or IHF, opens the DNA duplex in oriC and the DnaB helicase becomes inserted.
Fig. 3. SeqA stimulates in vitro replication at low DnaA concentrations. The indicated amounts of DnaA were added to replication reactions containing 180 nM SeqA (54 molecules per oriC) before incubation. The control contained the corresponding amount of SeqA buffer. The shown data points are average values obtained from two independent experiments; the range of variation between the two is <15 pmol for the four lowest values of DnaA. The inserted frame shows average stimulation by SeqA (180 nM) at 12 nM DnaA, obtained from five independent experiments. The error bars indicate the standard deviation.

To investigate whether SeqA affects replication before or after this stage, complexes were assembled on an oriC plasmid in the presence and absence of SeqA, and the resulting protein–oriC complexes were separated from unbound proteins by gel filtration (Materials and methods). Fractions from the gel columns were collected and assayed for replication activity by adding the rest of the replication proteins [primase, SSB, gyrase and DNA polymerase III (Pol III) with β-subunit]. Complexes assembled in the presence of SeqA exhibited a considerably reduced capacity for subsequent replication compared with complexes assembled in the absence of SeqA (Figure 4).

To verify that binding of SeqA is dependent on oriC, we compared the SeqA elution in parallel reactions containing either pBSoriC or the vector plasmid lacking oriC. Pre-priming complex mixtures containing pBSoriC or vector plasmid (both fully methylated) and SeqA, were incubated and gel filtered. In contrast to the case for pBSoriC, SeqA did not co-elute with non-oriC DNA; rather, SeqA eluted in later fractions (not shown). oriC nucleates binding of a large number of SeqA molecules: ~100% of the SeqA eluted in the DNA-containing fractions when present at 180 nM (54 SeqA molecules per oriC), and even at 560 nM (160 molecules per oriC) >80% of the SeqA eluted in these fractions.

**Effects of SeqA on the activity of pre-formed pre-priming complexes**

The interaction of SeqA with established pre-priming complexes was investigated as follows: pre-priming complexes were formed and gel filtered in the absence of SeqA, fractions were collected, and each fraction was split in two and incubated with either SeqA or control buffer. Replication activity was measured by adding the rest of the replication proteins. Again, SeqA strongly inhibited replication (Figure 6), showing that SeqA can act negatively even after the pre-priming complex is formed.
Effects of SeqA on replication in vitro

Fig. 5. A pre-priming complex is not formed in the presence of SeqA. The pre-priming complex mixture was incubated in the presence of 530 nM SeqA or the corresponding volume of SeqA buffer, and isolated by gel filtration through Biogel A15-m columns. (A) The upper panels show the levels of DnaA, DnaB, DnaC and SeqA proteins in the gel-filtered fractions (immunoblot analyses with antisera against DnaA, DnaB, DnaC and SeqA, respectively). The two lower panels show the amount of DNA recovered in each fraction (as a percentage of that loaded onto the column) and the amount of DNA synthesis (in pmol of nucleotides) obtained after adding the rest of the replication proteins to 20 μl of each fraction (see Materials and methods). (B) Quantification of the amount of DnaA, DnaB and DnaC proteins when added at this stage (Figure 7), in contrast to the significant reduction in protein levels detected when SeqA was present at pre-priming complex formation (Figure 5). The minor differences with and without SeqA seen in Figure 7 are within the experimental uncertainty in the immunoblotting and quantification procedures (see legend to Figure 7). Thus, SeqA is not able to displace significant amounts of proteins from an established pre-priming complex, indicating that the inhibitory effect on replication at this stage is due to mechanisms other than disruption of the pre-priming complex.

Discussion

SeqA can affect DNA replication at early stages in vitro

The experiments described here demonstrate that purified SeqA protein can affect DNA replication initiated from oriC in vitro in reactions containing pure replication components. These findings complement and extend earlier genetic evidence that SeqA affects DNA replication in vivo (Lu et al., 1994; von Freiesleben et al., 1994) and biochemical evidence that SeqA binds oriC in a methylation-sensitive manner (Slater et al., 1995).

The early stages of replication initiation are an orderly process including formation of an initiation-competent complex, then an open complex and finally a pre-priming complex. More specifically, DnaA protein binds to oriC and induces a localized unwinding of the AT-rich region that is proposed to be the entry site for DnaB helicase (Fuller et al., 1984; Bramhill and Kornberg, 1988). DnaA bound to oriC interacts with DnaB, complexed to DnaC, to permit binding of DnaB (Marszalek and Kaguni, 1994), thereby generating the pre-priming complex (Sekimizu et al., 1988a).
The current findings demonstrate that SeqA has the potential to influence one or more of these steps. In a fully reconstituted reaction, SeqA stimulates replication when DnaA protein is at limiting concentration (≤20 nM) and inhibits replication when the DnaA concentration is higher. Furthermore, in a staged reaction, SeqA inhibits formation of a pre-priming complex. Inhibition occurs at the concentrations of DnaA where inhibition of replication is observed in the reconstituted reaction, suggesting that these two effects are the same.

**SeqA can stimulate replication**

We favour the view that SeqA mediates its stimulatory effect by changing the distribution of DnaA molecules amongst the oriC regions present. At the DnaA concentrations where SeqA stimulates replication, the DnaA:oriC ratio is very low (<2:1 at 6 nM DnaA). Since DnaA binding to its five binding sites on linear oriC fragments is not co-operative (Messer et al., 1997), DnaA molecules are, in the absence of SeqA, probably distributed randomly amongst the available oriCs. Consequently, at low DnaA concentrations, relatively few oriCs contain enough DnaA molecules to undergo initiation. SeqA might exert its stimulatory effect by recruiting all of the DnaA molecules into functional initiation assemblies, with relatively little DnaA protein remaining at other sites, thus increasing the number of oriCs that are competent for initiation.

Alternatively, rather than changing the distribution of DnaA molecules on oriC templates, SeqA might instead increase the activity of the nucleoprotein complexes, thus permitting some of the ‘marginal’ complexes to be functional for early stages, via either direct and/or indirect effects of SeqA.

**SeqA can inhibit formation of a pre-priming complex**

Isolated oriC–protein complexes assembled in the presence of SeqA are defective in replication and contain SeqA protein in large amounts (~100 molecules per oriC); the DnaA, DnaB and DnaC contents of these complexes were reduced by 80–95% compared with complexes assembled without SeqA present. These findings could reflect effects of SeqA protein on initial assembly of DnaA onto oriC; alternatively, initiation-competent complexes may form but be unstable at later steps, e.g. during open complex formation. In either case, subsequent stable binding of DnaB and DnaC would be inhibited.

**SeqA can inhibit replication after formation of the pre-priming complex**

SeqA inhibits the standard reconstituted replication reaction when added together with the other proteins prior to incubation, i.e. before pre-priming complexes are formed. This inhibition is affected by the methylation status of the DNA template: at low levels of SeqA, only methylated oriC templates are inhibited. We infer that this reflects the specific binding of SeqA to fully methylated oriC. However, at higher concentrations of SeqA, replication of an unmethylated template is also inhibited. The fact that SeqA can act negatively, independently of the template methylation status, is in accordance with the in vivo observation that SeqA overproduction is lethal to methylation-deficient dam− cells (Lu et al., 1994).
Replication inhibition by SeqA also of an unmethylated oriC plasmid indicates that binding to oriC is no prerequisite for this mode of inhibition, suggesting that SeqA can inhibit replication by a direct interaction with the replication proteins. In accordance with this, we find that SeqA can inhibit replication also after the pre-priming complex is formed. This inhibition apparently occurs without the disruption of the pre-priming complex, as measured by the presence of its normal contents of DnaA, DnaB and DnaC proteins. We cannot exclude, however, that SeqA displaces these proteins from the complex at a later stage. Whether SeqA acts before or after Pol III has started replicating is not addressed in the present experiments. Hence, it is not clear whether SeqA inhibits progression from the pre-priming complex to the start of elongation, or inhibits elongation directly by interacting with the replication fork. However, preliminary data indicate that SeqA can inhibit in vitro replication at the elongation stage, in addition to an inhibitory effect occurring earlier in the initiation sequence (data not shown). Also, in vivo observations suggest that high levels of SeqA can inhibit elongation: flow cytometry experiments show that after strong SeqA overproduction, DNA replication is inhibited and the replication forks are stopped before they reach the terminus (our unpublished results).

**Implications for the role of SeqA in DNA replication in vivo**

In vivo, two different effects of SeqA on DNA replication have been distinguished thus far. First, SeqA delays replication initiation without perturbing the precision with which initiation is coupled to cell physiology (Boye et al., 1996); the precise step of replication initiation affected by SeqA in vivo is not known. Secondly, SeqA is required for sequestration of oriC from re-methylation immediately following replication initiation and, in a presumably related effect, for blocking replication of hemimethylated oriC plasmids (Lu et al., 1994).

How might the effects of SeqA observed in the current study be relevant to its in vivo roles? The in vivo effect of SeqA involves a change in the timing of the process, not on the probability that initiation will occur at all, whereas the current study has examined the effects of SeqA on the probability of occurrence of various steps. Also, staged reactions provide opportunities for effects that would, in an unpauased reaction, be unlikely on kinetic grounds. Nonetheless, despite these limitations, the findings presented here provide strong support for the notion that SeqA plays an integral role in replication initiation. Specifically, the data imply that SeqA modulates the assembly of an initiation-competent protein–DNA complex at oriC and then influences the behaviour of that complex once it has formed. Initiation complexes assembled in the absence of SeqA are capable of initiation but are probably defective with regard to more sophisticated behaviour such as replication timing and co-ordination, and sequestration. In this sense, SeqA might be considered a 'stringency factor', i.e. a component that constrains the initiation process to behave in accord with the available regulatory inputs. Accordingly, the observed SeqA-induced delay of initiation in vivo (Boye et al., 1996) may be a result of SeqA preventing the initiation process until the stage is set for a highly controlled and co-ordinated initiation. The delay may occur at any of the stages where SeqA effects have been observed: initial assembly, early stages or even exit of replication from oriC. Once the origin region itself has been replicated, SeqA presumably becomes bound to the newly hemimethylated oriC, perhaps together with residual DnaA molecules, to mediate sequestration.

**Materials and methods**

**Reagents**

Ribonucleoside triphosphates, deoxyribonucleoside triphosphates, polydl–dC–polydl–dC and Sephadex G-50 Nick columns were from Pharmacia. [γ-32P]ATP (>5000 Ci/mmol) and [ε-32P]dTTP (800 Ci/mmol) from Amersham; polyvinyl alcohol (mol. wt 30 000–70 000) and Igpal from Sigma; and 3-adenosyl methionine (SAM) from New England Biolabs.

**Plasmid DNA**

Plasmid pBSoriC (3640 bp), also called pTB101 (Baker and Kornberg, 1988), consists of a 678 bp Hincll–PstI fragment spanning oriC (~189 to +489 bp) cloned into the pBluescript vector.

The plasmids were purified by banding twice in CsCl–ethidium bromide density gradients as described (Sambrook et al., 1989), followed by desalting over Sephadex G-50 Nick columns. Unless otherwise stated, the plasmid used as template in replication reactions was fully methylated, i.e. grown in a wild-type strain (W3110). To eliminate the possibility of SeqA inhibiting replication by binding to persistent hemimethylated GATC sites on the DNA template, the experiment shown in Figure 1 was repeated with a template additionally methylated in vitro to ensure complete methylation at all sites. The additional in vitro methylation did not affect the inhibition by SeqA. The DNA template used in the experiments was, therefore, not additionally in vitro methylated, unless stated explicitly. In vitro methylation with Dam methylase and SAM was performed according to the manufacturer’s instructions, and followed by a second filtration through Sephadex G-50 Nick columns. Unmethylated pBSoriC was obtained from a dam strain (DS1310).

**Enzymes**

The gyrase B subunit and Pol III* were purified as described by Mizuuchi et al. (1984), and Maki et al. (1988), respectively. Primase and gyrase A subunit, provided by H.Nakai, were purified as described by Krulikis and Nakai (1994). DnaA protein, provided by E.Crooke, was purified as described by Sekimizu et al. (1988b). DnaB–DnaC in equimolar complex was provided by N.Dixon. SSB, β-subunit of DNA polymerase III holoenzyme and HU [purified as described by Kaguni and Kornberg (1984); Crooke (1995)] were a gift from A.Kornberg. IHF protein was a gift from H.E.Nash. The E.coli RNA polymerase was purchased from Pharmacia, and Dam methylase from New England Biolabs.

**Purification of SeqA**

We have used two preparations of SeqA. The purification of the first of these has been described before (Slater et al., 1995). The other preparation, which is functionally indistinguishable from the first, was purified by a procedure based on a protocol provided by T.Brendler (Brendler et al., 1995):

SeqA was overexpressed in E.coli strain B834 (DE3) harbouring pLYSs and pSS1, which is pET11a (Studier et al., 1990) with seqA under pT7 control (Slater et al., 1995). An inoculum of cells freshly transformed with pSS1 was grown in M9ZB medium (Studier et al., 1990) containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml). Growth was at 25°C, to improve solubility of SeqA. However, the solubility seems to be the same when grown at 37°C, so growing at higher temperature may be preferable. As the inducible production of SeqA was found to vary considerably, the response to isopropyl–[β–D-thiogalactopyranoside (IPTG) was checked by microscopy (the responding cells tend to become longer) in several parallel cultures after inducing with 0.4 mM IPTG for a few hours. A culture verified to respond to IPTG was diluted 1:500 into M9ZB (5 l) and grown to an OD600 of 0.45, when IPTG was added to 0.4 mM. The cells were grown with IPTG for 1–2 generations before harvesting. The cell pellets were resuspended in an equal weight of 250 mM potassium chloride, 25 mM HEPES–KOH pH 7.5, 10 mM magnesium acetate, 20 mM spermidine, 0.1 mM EDTA, 2 mM dithiothreitol (DTT) and 10% (v/v) sucrose. The cells were lysed by freezing.
and thawing, and the cell lysate centrifuged (45 min, 183,000 g, 1°C) in a Beckman 60Ti rotor. The supernatant contained very little SeqA and was discarded. SeqA was recovered from the pellet fraction by sonication in an equal volume of buffer [1 M KCl, 25 mM HEPES pH 7.5, 10 mM magnesium acetate, 20 mM spermidine, 2 mM DTT, 0.1% Igepal (v/v), 10% (w/v) sucrose]. The sonicated pellet was centrifuged (45 min, 183,000 g, 1°C). The supernatant (Fraction I, 10.2 ml) was removed and the proteins precipitated with 0.35 g/ml ammonium sulfate. The precipitate was dialysed in 300 mM ammonium sulfate, 40 mM HEPES pH 7.5, 1 mM magnesium acetate, 0.1 mM EDTA, 2 mM DTT, 0.1% Igepal (v/v) and 15% (v/v) glycerol (Fraction II, 2 ml). Fraction II was diluted to a final salt concentration of 200 mM ammonium sulfate (with little loss of protein), loaded onto a heparin–ammonium sulfate, added SDS loading buffer. In the experiments when immunoblot analysis of the filtered fractions was performed at room temperature through Biogel A15-m: a second stage contained DNA template, DnaA, DnaB, DnaC and HU pre-priming buffer, and five-drop fractions (~70 μl aliquot of each fraction was used for the replication reaction. The rest of the fraction was added SDS loading buffer. In the experiments when immunoblot analysis of the filtered fractions was performed at room temperature through Biogel A15-m: 15 μg of primase, 88 μg of Pol III, 8 ng of subunit of Pol III, 8 ng of the subunit of Pol III, 8 μg of the subunit of Pol III; 3 and 1) or IHF, and 64 ng of DnaA unless otherwise stated, and dATP, dGTP, dCTP and dTTP each at 0.1 mM, with [α-32P]dTTP at 30–200 c.p.m. per pmol of deoxyxynucleotides. All data shown are obtained from RNA polymerase-independent reactions. The RNA polymerase-dependent reaction (25 μl) additionally contained UTP, GTP and CTP at 0.5 mM, 100 ng of Hu and DNA polymerase at the indicated amounts. DnaA protein was added at the indicated amounts.

Mixtures were assembled at 0°C, incubated at 29°C for 20 min and then precipitated with 500 μl of cold 10% trichloroacetic acid containing 100 mM PPi. Total nucleotide incorporation was measured by liquid scintillation counting after filtration onto GF/C glass-fibre filters.

Replication reaction started at pre-priming complex formation

The first stage contained DNA template, DnaA, DnaB, DnaC and Hu or IHF (in the same amounts as above) in 20 μl of pre-priming buffer [50 mM Tricine–KOH (pH 8.2), 5 mM ATP, 0.25 mg/ml bovine serum albumin, 0.01% (v/v) Brij 58, 0.3 mM EDTA, 60 mM potassium glutamate and 20% (v/v) glycerol]. Incubation was at 29°C for 10 min. The pre-priming complexes thus formed were gel filtered. The gel filtering was performed at room temperature through Biogel A15-m: 100 μl of pre-priming mixture was loaded onto one ml columns equilibrated with pre-priming buffer, and five-drop fractions (~70 μl) were collected. The rest of the replication proteins were added to the fractions in the amounts described above and the mixture incubated at 29°C for 20 min. A 20 μl aliquot of each fraction was used for the replication reaction. In the experiments when immunoblot analysis of the filtered fractions was performed, the rest of the fraction was added SDS loading buffer.

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