Early dissociation of nuclear factor I from the origin during initiation of adenovirus DNA replication studied by origin immobilization

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ABSTRACT

The DNA-binding domain of Nuclear Factor I (NFIBD) enhances initiation of adenovirus DNA replication up to 50-fold by binding to the auxiliary region of the origin and positioning the viral DNA polymerase. To study if and when NFIBD dissociates from the template, we immobilized origin DNA to glutathione–agarose beads by means of a GST–NFIBD fusion protein. This immobilized template is active in replication. By analyzing the release of prelabeled templates from the beads under different conditions, we show that NFIBD dissociates already early during initiation. During preinitiation NFIBD remains bound, but as soon as dCTP, dATP or dTTP are added, efficient dissociation occurs. A much lower dissociation level was induced by addition of dGTP. Since dCTP, dATP and dTTP are required for formation of a pTP-CAT initiation intermediate, we explain our results by conformational changes occurring in the polymerase during initiation leading to disruption of both the interaction between the polymerase and NFI as well as the interaction between NFI and the DNA.

INTRODUCTION

The Ad5 genome consists of a linear 36 kb double stranded DNA molecule with a terminal protein (TP) covalently attached to both 5'-terminal dC-residues. Two identical origins of replication, each composed of a core region encompassing the terminal 18 base pairs and an auxiliary region spanning the adjacent 30 nucleotides, are present at the molecular ends. Replication can be reconstituted *in vitro* employing purified recombinant proteins. Three viral proteins are absolutely required for replication. These are the DNA polymerase (pol) and the precursor of the terminal protein (pTP), which form a tight complex (pTP-pol), as well as the Ad DNA-binding protein (DBP). Two cellular transcription factors, nuclear factor I (NFI) and Oct-1, play a role as accessory factors, enhancing the number of initiation events by increasing the V_{max} of the reaction (1). NFI binds as a dimer to its recognition sequence in the Ad5 origin and stimulates initiation up to 50-fold

at low concentrations of the pTP-pol complex (2–4). Oct-1 stimulates initiation up to 7-fold (5–7), also depending on the pTP-pol concentration (4,8). All activities associated with the ability of NFI and Oct-1 to stimulate Ad replication are confined to their conserved DNA-binding domains, NFIBD and the Oct-1 POU domain (2,3,7,9).

Based on reconstitution experiments the events taking place during the initiation reaction can be depicted as follows (reviewed in 10–12). The first step in the initiation reaction involves the assembly of a preinitiation complex. A TP-DNA template forming a multi-protein–DNA complex with DBP, functions as a starting point for complex assembly. The crucial event in preinitiation complex formation is the association of the pTP-pol complex with the viral template. Via interactions with NFI and Oct-1, the pTP-pol complex is recruited to the origin and correctly positioned at the core region (4,8,9,13). Correct binding of the pTP-pol complex is further achieved by recognition of the core origin aided by the parental TP (13–17). The function of DBP in preinitiation complex formation is to increase the affinity of NFI binding to its recognition site in the Ad *ori* (18,19). After the assembly of a preinitiation complex, pTP-pol catalyzes the actual initiation reaction, i.e. the formation of a pTP-trinucleotide (CAT) complex (20).

Although the role of NFI in preinitiation is fairly well defined, it is not clear if and when the protein dissociates from the stably bound template (21). Since this problem is difficult to study using the soluble replication assay, in which NFI is present in excess, we developed an immobilized system for Ad DNA replication and used this to study dissociation.

MATERIALS AND METHODS

Construction of expression vectors

The N-terminal DNA-binding domain of rat liver NFI (NFIBD) containing amino acids 5–240 (3) was cloned into the *Bam*HI site of expression plasmid pRP261. This plasmid is a derivative of pGEX-3X (22). Isopropyl β -D-thiogalactopyranoside (IPTG) induction of the *tac*-promoter results in synthesis of a 467 amino acids fusion protein containing a functional GST part (amino acids

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1–228, containing an A₂₂₄G mutation and a factor X_a site [a.a. 222–225]), an aspecific linker region (a.a. 229–231, A–G–R) and NFIBD amino acids 5–240 (a.a. 232–467).

Expression and purification of fusion proteins

GST–NFIBD was expressed in *E. coli* strain BL21(DE3). For optimal expression of fusion proteins overnight cultures grown at 37°C were diluted 1:100 in LB medium in the presence of 0.4% glucose and 100 µg/ml ampicillin and allowed to re-enter log phase. After 90 min of vigorous shaking (OD₆₀₀ = 0.5) 0.4 mM IPTG was added followed by 2 h of induction. Bacteria were then harvested by centrifugation and resuspended in lysis buffer (20 ml per liter culture) containing 50 mM Tris–HCl pH 7.9, 1 mM EDTA, 250 mM NaCl, 5 mM dithiothreitol (DTT), 5 mM Na₂S₂O₅ and 350 µg/ml lysozyme. Incubation was for 30 min at room temperature followed by a freeze/thaw step in the presence of 0.1% NP-40 and ultrasound sonification. The resulting extract was treated with DNase I (20 µg/ml) for 45 min at room temperature. Soluble proteins were separated from inclusion bodies and bacterial debris by centrifugation at 12 000 r.p.m. in a Sorvall ss34 rotor for 20 min at 4°C.

Since virtually no GST–NFIBD was present in the soluble fraction we purified the fusion protein from the bacterial inclusion bodies as described in (8). Renatured proteins were applied to a DEAE column equilibrated with renaturation buffer. The flowthrough of this column was loaded directly onto a glutathione–agarose (GA; Sigma) affinity column. One ml (80 mg dry-weight) of GA efficiently bound 5 mg fusion protein. This column was washed extensively with ten column volumes of buffer A, containing 150 mM NaCl, 20 mM NaPO₄ pH 7.5, 1 mM DTT, 1% Triton X-100 pH 7.3 and 15% (v/v) glycerol. GST-fusion proteins eluted efficiently using buffer B/100 mM NaCl in the presence of 5 mM reduced glutathione (Sigma). Buffer B contains 50 mM Tris–HCl pH 8.0, 1 mM DTT, 5 mM Na₂S₂O₅, 15% (v/v) glycerol and 0.5 mM PMSF. To remove glutathione and bring the renatured GST–NFIBD preparation to homogeneity, peak activities were applied to a DNA recognition site affinity column equilibrated in buffer B/100 mM NaCl. This column is prepared from a plasmid (pKB67-88) that contains 88 copies of Ad bp 1–67 including the NFI recognition site (23). After extensive washing, GST–NFIBD was eluted stepwise using buffer B/500 mM NaCl. Protein integrity, concentration and purity were determined by Coomassie brilliant blue staining after SDS–PAGE.

Gel retardation assay

The 110 bp *EcoRI*–*XbaI* fragment from pHRI, containing the Ad2 origin was labelled at the *XbaI* end with DNA polymerase I (*Klenow* fragment) in the presence of 100 µM dATP, dGTP and dTTP, as well as 50 µCi α³²P-dCTP (3000 Ci/mmol) per microgram plasmid DNA. This probe was incubated in 25 mM HEPES–KOH (pH 7.5), 0.4 mM DTT, 4% (w/v) Ficoll and 150 mM NaCl with various amounts of protein (as indicated) in a total volume of 25 µl for 1 h at 4°C. Bound and free DNA were separated on a 6% polyacrylamide gel in running buffer containing 45 mM Tris, 45 mM boric acid, 1 mM EDTA and 0.01% NP-40.

Development of an immobilized system to study the initiation of Ad DNA replication

Pre-swollen GA beads were coated with 100 µg/ml casein to prevent aspecific binding. Subsequently, for ten incubations 2

µg GST–NFIBD was allowed to bind for 15 min on ice and the beads (100 µl; 50% v/v) were loaded onto a 1 ml column, allowed to pack at room temperature and washed with 5 ml of buffer C, containing 25 mM HEPES–KOH (pH 7.5), 1 mM MgCl₂ and 0.4 mM DTT, in the presence of 50 mM NaCl (buffer C/50 mM NaCl). In the next step, 5 µg *XhoI* digested Ad5 TP-DNA was applied to this column and recycled for 60 min at room temperature. Digestion of the Ad5 genome with *XhoI* results in seven DNA fragments (A–G) of which the B and C fragments (6200 and 5700 bp, respectively) contain the Ad origin of replication covalently linked to the terminal protein. Alternatively, we added 4 µg of an (end-labelled) *EcoRI*–*AvaII* digest of plasmid pHRI. Digestion of this plasmid results in three DNA fragments of which the largest fragment contains the Ad *ori*. DNA was allowed to bind to GST–NFIBD under a constant flow of 15 column volumes/hour in buffer C containing 150 mM NaCl to avoid aspecific binding of GST–NFIBD to the DNA. Beads were collected and any remaining unbound DNA was washed away using buffer C/50 mM NaCl. If part of the GST–NFIBD population would bind DNA aspecifically at 50 mM NaCl due to incorrect refolding, such DNA would be removed under these NaCl conditions (see Fig. 3B). Next, beads were resuspended in 100 µl buffer C/50 mM NaCl and 10 µl aliquots were transferred to an eppendorf vial and used as a template. Reaction mixtures (25 µl) contained different combinations of replication proteins (as indicated), as well as 40 µM of [dATP, dGTP and dTTP] and 250 nM of α³²P-dCTP (600 Ci/mmol) in buffer C/50 mM NaCl. The suspensions were incubated for 60 min at 37°C on a tumbling-wheel and the reactions were stopped by immediate cooling on ice, followed by addition of 2.5 µl of stopmix (40% sucrose, 1% sodium dodecyl sulphate [SDS], 0.1% bromophenol blue and 0.1% xylene cyanol). The reaction products were analyzed by agarose gel electrophoresis in the presence of 0.1% SDS. These gels were partially dehydrated and autoradiographed.

NFI dissociation assay

The dissociation of NFIBD from the template can be monitored by the release of radiolabeled DNA fragments. In order to measure dissociation of the template, initially the following assay was developed. GST–NFIBD (1 µg for five incubations) was immobilized to 25 µl casein coated GA beads (50% v/v) in buffer C/150 mM NaCl. After three 500 µl wash steps, beads were incubated with 2 µg of the pTP-pol complex, 5 µg of DBP and 2 µg of prelabeled pHRI × *EcoRI* × *AvaII* in buffer C/100 mM NaCl. Immobilized preinitiation complexes were spun down, washed four times using buffer C/50 mM NaCl and resuspended in 100 µl of the same buffer; 20 µl aliquots were incubated with different combinations of dNTPs as indicated. After 30 min at 37°C, the beads were spun down by short (< 15 s) centrifugation in a microfuge. To the supernatant (25 µl) 2.5 µl stopmix was added and the products were analyzed as described. This method—used in Figure 4A—was later improved by allowing the formation of (pre-)initiation complexes prior to addition of GA beads (Fig. 4B and C). Here, assembly was allowed to proceed for 30 min at 37°C after combining 400 ng of TP-free template molecules (pHRI × *EcoRI* × *AvaII*), 400 ng of pTP-pol, 400 ng DBP and 200 ng of GST–NFIBD in buffer C/100 mM NaCl. Next, to each incubation 5 µl of casein coated GA beads were added and immobilization was allowed to proceed for 15 min on ice. This step favoured the association of DBP molecules with the template, thereby reducing aspecific binding of sticky

DBP molecules to the agarose matrix. Finally, the beads were washed, resuspended and dissociation reactions were performed in the presence of nucleotides, or nucleotide-analogues (as indicated). Samples were treated further as described above.

RESULTS

Renatured GST–NFIBD is active in reconstituted Ad DNA replication

After renaturation 10% of the total protein input is retained by the GA column (Fig. 1A). As shown in this figure, the full-length fusion protein was the major species (more than 90%) present in the preparation. Employing bandshift assays we show that renatured and GA-purified GST–NFIBD binds to the NFI recognition sequence (Fig. 1B). Of the large amount of protein not binding to the glutathione coated beads, only a minimal amount is active in DNA binding, indicating that in these molecules both the GST part as well as the NFIBD part are incorrectly folded. After the DNA affinity column, the overall yield of active GST–NFIBD was ~5%. Comparison of equimolar amounts of GST–NFIBD to recombinant vaccinia virus expressed NFIBD in the reconstituted Ad DNA replication system (Fig. 1C) shows that the latter is approximately five times more active than its *E. coli* expressed counterpart; this is due to the 5-fold lower binding activity of *E. coli* GST–NFIBD. As determined by bandshift assays, one binding unit (as defined in 4) of GST–NFIBD equals ~15 ng and one binding unit of vaccinia NFIBD corresponds to 1.4 ng. After correction for the GST moiety this means a 5-fold difference in binding activity.

Immobilized GST–NFIBD binds both pTP-pol and stimulates replication

In order to determine the feasibility of an immobilized replication assay, we first tested if immobilized GST–NFIBD could bind pTP-pol and stabilize a preinitiation complex similar as soluble NFIBD. We preincubated immobilized GST–NFIBD with an *Xho*I digest of Ad5 DNA or with the pTP-pol complex, followed by extensive washing. Next, we allowed replication by adding all remaining components as indicated, including labeled dCTP. Specific replication of origin containing B and C fragments was observed when GST–NFIBD was used (Fig. 2A, lanes 1 and 2), but not with control GST alone (lanes 3 and 4). Even some labeled single stranded B and C fragments were formed, indicating second rounds of replication. Both DNA (lane 1) as well as the pTP-pol complex (lane 2) are able to interact with immobilized GST–NFIBD permitting specific replication. This indicates that the NFIBD moiety is active similar as soluble NFIBD. A relatively high amount of intermediate replication products was observed in lane 2, in the absence of excess polymerase during replication. The presence of these intermediates probably reflects dissociation of the polymerase during the elongation reaction, resulting in unfinished replication products. In a second experiment (Fig. 2B), we analyzed whether the interaction between NFIBD and the pTP-pol complex is influenced by the presence of DNA and DBP during preincubation (lanes 2 and 3). This was not the case, showing that association of NFI with the pTP-pol complex can occur prior to and independent of the DNA recognition step, which is in agreement with the results obtained in solution (9,13,15). Apparently, DBP remained bound in sufficient amounts to enable replication (lane 3).

Prelabeled immobilized templates can be used for dissociation studies

Since analysis of the dissociation of NFI requires the use of prelabeled DNA fragments, we assayed the conditions under which immobilized GST–NFIBD can bind these origin-containing DNA fragments specifically. GST–NFIBD was bound to GA beads and an end-labeled *Xho*I digest of the natural TP-DNA template was added. Specific binding of origin-containing B and C fragments was optimal at 150 mM NaCl. At this concentration, only a small amount of non-origin containing fragments were bound, whereas at lower NaCl concentrations all other internal fragments were bound effectively (Fig. 3A). Addition of NaCl concentrations of 200 mM and more

Figure 1. Renatured GST–NFIBD binds DNA and stimulates Ad DNA replication in solution. (A) Glutathione–agarose affinity chromatography of renatured protein samples analyzed by Coomassie-stained SDS–PAGE gels. M = molecular weight (kDa) marker; L = load. Flowthrough (FT) fractions 1–7 contain inactive, presumably non-renatured fusion proteins. S = glutathione step elution (B) Analysis of the same fractions by gel retardation. F = free probe (no protein added); P = probe; C = protein–DNA complex. Of each column fraction 5 ng of protein was incubated with 3 fmol (10 000 c.p.m.) of the 110 bp *Eco*RI–*Xba*I fragment from pHRI. (C) Stimulation of Ad DNA replication *in vitro*. Replication was studied under standard conditions (28) without (–) or with increasing amounts of NFIBD. For vaccinia expressed NFIBD we added 0.5, 1, 2 and 4 ng of protein, for GST–NFIBD we tested 1, 2, 4 and 8 ng (equimolar) of purified protein. B/C refers to the replicated origin-containing fragments, ssB/C to the topstrands of these fragments which were synthesized in the first round of replication and displaced during the second round of replication. I = intermediate replication forms.

resulted in an overall decrease in binding affinity. At 150 mM NaCl ~2% of the input DNA is bound. To improve this level higher concentrations of origin DNA are required. However, this is difficult to achieve using TP containing-DNA since standard biochemical techniques—e.g. precipitation of DNA using ethanol or 2-propanol—denature the terminal protein, rendering the template inactive. This same reason thwarts the removal of dNTPs and enzymes added during the cleavage and labelling reactions. Therefore, we employed TP-free templates, obtained from an *EcoRI/AvaII* digest of origin-containing plasmid pHRI. As shown in Figure 3B, lanes 4 and 5, between 150 and 200 mM NaCl binding of the non *ori*-containing fragments (222 and 1023 bp) drops below 5%, while still ~20% of the *ori*-containing fragment (1530 bp) binds to GST–NFIBD, 10-fold more than with TP-DNA (Fig. 3A). Importantly, the immobilized TP-free template is able to replicate (data not shown). The efficiency of template usage was ~1%, which is comparable to the efficiency in solution.

Dissociation of NFIBD from the Ad *ori* occurs prior to elongation

In order to investigate the moment of dissociation of NFI we measured the release of prelabeled TP-free DNA bound to GA

Figure 2. Immobilized GST–NFIBD binds DNA and pTP-pol independently. (A) Immobilized GST–NFIBD binds DNA and interacts with the pTP-pol complex. Immobilized GST–NFIBD (200 ng) was incubated with 400 ng of the pTP-pol complex and 500 ng Ad5 $\times$ *XhoI* ('DNA' in the figure) when indicated. This mixture was incubated for 30 min on ice followed by a stringent washing procedure. Next, replication reactions (25 μ l) were allowed to proceed in the presence of DBP (200 ng) and dNTPs (NT) When not present during preincubation, DNA and pTP-pol were added during the replication reaction. Since only 2% of the template input is bound, we decreased the amounts of DNA and pTP-pol added in this latter incubation to 25 and 20 ng, respectively. Lanes 1 and 2 contain immobilized GST–NFIBD. Lanes 3 and 4 immobilized GST. (B) The NFI–pTP-pol interaction is not significantly stabilized by template DNA or DBP. Preinitiation complex formation was allowed as described for panel A. Lane 3 contained 2 μ g of DBP during preinitiation.

beads via GST–NFIBD under various conditions. This approach was feasible since the NFI–DNA interaction is rather stable and barely any DNA dissociates spontaneously. We assayed dissociation during four well-defined stages in viral replication. As shown in Figure 4A (lane 1), upon preinitiation complex formation only a limited amount of labeled origin fragments dissociate from the assembled preinitiation complexes (less than 5%). Next, we incubated the immobilized preinitiation complexes with dCTP and ddATP, leading to pTP–dCMP formation (lane 2). Under these conditions up to 20% of all bound templates are dissociated. Further addition of dATP, dTTP and ddGTP (formation of a pTP-26 nucleotides complex) did not increase the dissociation level, whereas addition of dATP, dTTP and dGTP (full length elongation) slightly increased the dissociation level (to 25%).

We also investigated dissociation of TP-containing B and C bands. Despite the low detection levels due to the inefficient binding of TP-DNA to immobilized GST–NFIBD a similar result was obtained, i.e. the DNA–NFI interaction was already disrupted upon initiation (data not shown).

Dissociation of NFIBD is effected by the pTP-pol complex in the presence of nucleotides

We were surprised that dissociation occurred already so early, since the NFI binding site is presumably still double stranded at this stage. Moreover, dissociation levels are much higher than replication levels, thus no direct correlation with initiation is found. To investigate this in more detail, we repeated the experiment under conditions in which the dissociation background level was further lowered (see M. & M. and Fig. 4B, lane 1). As shown in Figure 4B, just the addition of several dNTPs—in the presence of pTP-pol—led to considerable dissociation (up to 50%) of the bound DNA. The level of dissociation is concentration dependent, maxima being reached at 40 μ M.

Figure 3. DNA binding profile of GST–NFIBD as a function of the NaCl concentration. 500 ng Ad5 DNA $\times$ *XhoI* (A) or 400 ng origin-containing plasmid pHRI $\times$ *EcoRI* $\times$ *AvaII* (B) were labeled by *Klenow* fragment in the presence of α^{32} P–dCTP and incubated in buffer C with 200 ng of immobilized GST–NFIBD at different NaCl concentrations. This amount of GST–NFIBD can bind 12 fmol of pHRI (200 ng) or 6 fmol of Ad5 (1400 ng) when assayed in solution.

Interestingly, dGTP was the least efficient inductor, dissociating less than 10%. This may be related to our recent observation that a pTP-CAT intermediate is formed during initiation (King and Van der Vliet, in press). Apparently, the dissociation of NFI does not require the actual initiation reaction, but takes place at some stage between preinitiation complex formation and pTP-CAT formation.

We further investigated the requirements for dissociation by incubating the preinitiation complex with dCDP, dCMP or ATP. As shown in Figure 4C, dissociation is strongly enhanced in the presence of dCDP and dCTP (lanes 4–7), but only very limited after addition of dCMP and ATP (lanes 2, 3 and 8, 9). As an important control, we tested whether addition of dNTPs had a direct effect on NFI–DNA complex formation employing bandshift assays; this was not the case (not shown). We interpret these results to mean that the complex between NFI and pTP-pol, as well as that between NFI and DNA, is disrupted by a conformational change in the polymerase possibly accompanied

by a subsequent change in the conformation of the NFI binding site. The former change can be induced by the presence of nucleotides.

DISCUSSION

Applying origin immobilization, we studied the dissociation of NFI from its recognition sequence in the Ad2/5 *ori*. A priori, we figured that dissociation would occur during elongation as a consequence of the passing polymerase, unwinding the dsDNA NFI recognition sequence. However, we observe that NFI dissociates much earlier, directly after the formation of a stable preinitiation complex (Fig. 4). Dissociation takes place in the presence of dCTP, but does not require the actual initiation reaction since dissociation can be induced efficiently by dATP, dCDP, dCTP and dTTP. This observation is possibly correlated to recent findings showing that pTP-CAT, rather than pTP-C, is the intermediate formed during the initiation reaction (20). Possibly the polymerase, when in the initiation mode, preferably binds dATP, dCDP, dCTP and dTTP, explaining why dGTP is not very effective in the induction of NFI dissociation. We hypothesize (Fig. 5) that binding of dATP, dCDP, dCTP and dTTP induces a conformational change in the polymerase, which will subsequently lead to disruption of the NFI–pol interaction. Simultaneously, the polymerase induces structural changes in the origin, e.g. unwinding, resulting in dissociation of NFI from the DNA. The polymerase remains bound to the open origin, keeping the NFI site inaccessible (Fig. 5). This activated preinitiation complex is able to allow pTP-CAT formation. Activation events of preinitiation complexes formed on non-origin containing DNA

Figure 4. Dissociation of NFIBD from the (prelabeled) template is effected by the polymerase after addition of nucleotides. (A) Dissociation of NFIBD from TP-free DNA occurs prior to elongation. The input lane contains a 20 μ l aliquot of the immobilized 32 P-prelabeled template. Lane 1 = 10 mM Tris–HCl pH 8.0. Lane 2 = dCTP and ddATP. Lane 3 = dCTP, dATPdTTP and ddGTP. Lane 4 = dCTP, dATP, dGTP and dTTP. Each d(d)NTP was added to a final concentration of 40 μ M. (B) Dissociation of NFIBD from the Ad *ori* is effected by addition of nucleotides. Lane 1 = 10 mM Tris–HCl pH 8.0. Lanes 2, 3 = dCTP. Lanes 4, 5 = dGTP. Lanes 6, 7 = dATP. Lanes 8, 9 = dTTP. Lane 10 = 10% of the input. dNTPs were added to final concentrations of 2 μ M (even numbers) or 40 μ M (odd numbers). Note that background levels are reduced as a consequence of the reduction of aspecifically bound fragments (see M. & M.). (C) Lane 1 = input (50%). Lanes 2, 3 = dCMP. Lanes 4, 5 = dCDP. Lanes 6, 7 = dCTP. Lanes 8, 9 = ATP. Lane 10 = 10% of the input. Nucleoside phosphates were added to final concentrations of 1 μ M (even numbers) or 40 μ M (odd numbers).

Figure 5. Mechanism of NFI dissociation. To account for the obtained results we propose the following sequence of events. The NFI recognition sequence is indicated in black. After formation of an immobilized preinitiation complex (A), addition of specific dNTPs results in the induction of a conformational change of the polymerase (B; activated preinitiation complex). This change leads to dissociation of NFI (see text), possibly accompanied by a shift in the position of the polymerase towards the conserved sequence element ranging from bp 9–18 (dark shading). Hereafter the activated polymerase initiates Ad DNA replication from an unwound origin (C).

are abortive since the polymerase does not encounter the specific association sequence. This model adequately explains why the dissociated template molecules do not re-associate with the pool of 'free' GST-NFIBD.

We have shown that 50% of the templates immobilized by GST-NFIBD dissociate after addition of nucleotides (Fig. 4B and C). Since dissociation is effected by the polymerase, this means that at least 50% of all immobilized templates assembled into preinitiation complexes. Interestingly, in spite of this observation, only 1% of the immobilized template molecules actually replicates. This paradox might be explained by the occurrence of a rate-limiting step after the formation of an activated preinitiation complex. Since we have previously shown (1) that no rate-limiting steps occur after the actual initiation event (i.e. pTP-CAT formation) we suggest that opening of the origin might be the rate-limiting step. The changes in the origin structure accompanying the unwinding reaction—required for initiation—might easily result in dissociation of the polymerase, promoting polymerase idling.

We observed that DNA-binding by immobilized GST-NFIBD is less specific than measured for the soluble form. At NaCl concentrations below 150 mM, immobilized GST-NFIBD loses the ability to discriminate between the origin-containing B and C fragments and the aspecific A, D, E and F fragments (Fig. 3A), whereas GST-NFIBD—in solution—specifically stimulates replication of B and C fragments at 50 mM NaCl (Fig. 1C). This decreased specificity presumably reflects the influence of immobilization per se. One of the properties of NFI that is possibly influenced by immobilization is the ability to dimerize, since GST-NFIBD dimers, essential for high affinity, as well as specific DNA-binding (2,3), might be affected by the very strong GA-GST interaction. However, immobilized templates are able to replicate, licensing the use of this system in the study of events preceding actual replication.

At 100 mM NaCl GST-NFIBD can recognize both the origin-containing 1530 bp fragment, as well as the 1023 bp fragment, which presumably contains a cryptic NFI binding site (Fig. 3B). Importantly, when pTP-pol and dNTPs are added also the non-origin containing fragment is released (data not shown). Therefore, we stress that polymerase-induced dissociation of NFI occurs irrespective of the source of the template. This forms an additional indication that dissociation occurs prior to the initial pTP-CAT formation, which is origin-dependent (20).

Dissociation of GST-NFIBD occurred only after addition of specific nucleotides. A comparable situation is observed in the kinetic scheme of nucleotide incorporation by *Klenow* fragment of *E.coli* DNA polymerase I (24). Here, binding of dNTP to the polymerase-DNA complex is followed by a change in the polymerase conformation from the open to the closed state. This change is dependent on the presence of a DNA template and occurs only after binding of a correct dNTP (for review see 25). Another recently elucidated example is provided by the *E.coli* Rep helicase, for which both ADP and ATP serve as allosteric effectors of DNA binding (26).

An interesting parallel to the immobilized system is presented by the recent observation made by Sun *et al.* (27) that NFI interacts with the inner nuclear membrane *in vivo*.

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