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Host proteins can stimulate Tn*7* transposition: a novel role for the ribosomal protein L29 and the acyl carrier protein

Pamela L.Sharpe and Nancy L.Craig[1]

The Johns Hopkins University School of Medicine, Department of Molecular Biology and Genetics, Howard Hughes Medical Institute, 725 North Wolfe Street, Room 601 PCTB, Baltimore, MD 21205, USA

[1]Corresponding author
e-mail: nancy_craig@qmail.bs.jhu.edu

The bacterial transposon Tn*7* is distinguished by its ability to insert at a high frequency into a specific site in the *Escherichia coli* chromosome called *attTn7*. Tn*7* insertion into *attTn7* requires four Tn*7*-encoded transposition proteins: TnsA, TnsB, TnsC and TnsD. The selection of *attTn7* is determined by TnsD, a sequence-specific DNA-binding protein. TnsD binds *attTn7* and interacts with TnsABC, the core transposition machinery, which facilitates the insertion of Tn*7* into *attTn7*. In this work, we report the identification of two host proteins, the ribosomal protein L29 and the acyl carrier protein (ACP), which together stimulate the binding of TnsD to *attTn7*. The combination of L29 and ACP also stimulates Tn*7* transposition *in vitro*. Interestingly, mutations in L29 drastically decrease Tn*7* transposition *in vivo*, and this effect of L29 on Tn*7* transposition is specific for TnsABC + D reactions.

Keywords: acyl carrier protein (ACP)/ribosomal protein L29/target site-selection/transposon Tn*7*

Introduction

Transposable elements are discrete DNA segments that can translocate between sites within a genome. Although transposable elements can usually insert into many different sites, most transposons do exhibit some degree of target-site selectivity (reviewed by Craig, 1997). The bacterial transposon Tn*7* is unusual in that it can utilize two distinct classes of target sites using two different transposition reactions. Using one class of target sites, Tn*7* inserts at a high frequency into a specific site called *attTn7* found in the chromosomes of many bacteria; alternatively, Tn*7* also inserts at a low frequency into another class of non-*attTn7* sites (Barth *et al.*, 1976; Lichtenstein and Brenner, 1981; Craig, 1991; Wolkow *et al.*, 1996).

Multiple Tn*7*-encoded proteins, TnsA, TnsB, TnsC and TnsD (TnsABC+D), mediate Tn*7* transposition into *attTn7* (Rogers *et al.*, 1986; Waddell and Craig, 1988; Kubo and Craig, 1990; Bainton *et al.*, 1993). TnsD, a sequence-specific DNA-binding protein, is responsible for selecting the *attTn7* target site (Waddell and Craig, 1988; Kubo and Craig, 1990; Bainton *et al.*, 1993) and subsequently for recruiting the core transposition machinery to the target DNA. This machinery is a oligomeric complex containing both TnsA and TnsB, (May and Craig, 1996; Sarnovsky *et al.*, 1996) and TnsC, the transposition regulator (Stellwagen and Craig, 1997). Thus, the role of TnsD in Tn*7* transposition is to position the core transposition machinery at the Tn*7* insertion site.

The binding of TnsD to *attTn7* exerts considerable control over Tn*7* transposition into *attTn7*. Previous studies have demonstrated that the initiation of the chemical steps, i.e. cleavage and joining steps, of the transposition reaction requires the recognition of *attTn7* by TnsD. We have previously proposed that cleavage at the Tn*7* ends occurs only after the formation of a nucleoprotein complex containing TnsABC+D, *attTn7* target DNA and Tn*7* ends (Bainton *et al.*, 1991, 1993). Although the selection of an appropriate target DNA takes place early in Tn*7* transposition reaction, not all transposons do so at an early step. In contrast to Tn*7*, Tn*10* selects its target sites at a late step in the transposition reaction; the Tn*10* transposase does not interact with or capture the target DNA until the complete excision of the Tn*10* element (Sakai *et al.*, 1995; Kleckner *et al.*, 1996; Sakai and Kleckner, 1997). Regardless of the stage at which the target DNA enters the transposition reaction, the interaction of the transposase with appropriate DNA targets is a vital step in transposition.

Host-encoded proteins can also influence target-site selection. For example, the recruitment of the yeast retrovirus-like Ty3 element to its preferred insertion sites located within the promoters of genes transcribed by RNA polymerase (pol III), occurs through the direct interaction of the Ty3 integrase and the pol III transcription factors TFIIIB and TFIIIC (Kirchner *et al.*, 1995). Do host proteins also influence the target-site selectivity of Tn*7*? There has been no obvious requirement for host proteins in Tn*7* transposition. Indeed, efficient reconstitution of Tn*7* transposition *in vitro* has been accomplished using highly purified Tn*7*-encoded transposition proteins (Bainton *et al.*, 1993). However, it has been observed in previous work that the addition of crude extract to the transposition reaction can stimulate Tn*7* transposition *in vitro* (Bainton *et al.*, 1991) and more specifically that host proteins can stimulate TnsD binding to *attTn7*, suggesting that host proteins may be involved in Tn*7* transposition (Bainton *et al.*, 1993).

To determine the identity and the roles of the host proteins that are involved in stimulating TnsD binding to *attTn7* and potentially Tn*7* transposition, we fractionated host-cell extracts using TnsD binding to *attTn7* as an assay. Two host proteins, the ribosomal protein L29 and the acyl carrier protein (ACP), were identified that collaborate to assist the formation of TnsD–*attTn7* complexes. L29 is a component of the 50S ribosomal subunit and can bind to 23S rRNA (Urlaub *et al.*, 1995; Wittmann-

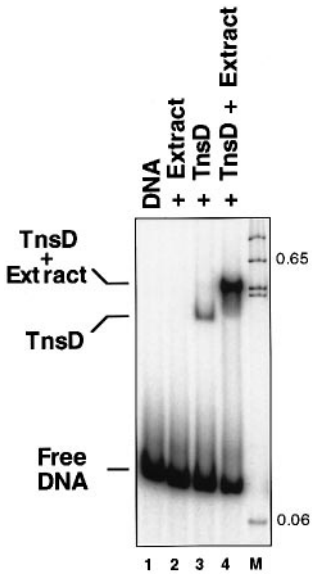

Fig. 1. Evaluation of host crude extracts for their ability to alter TnsD binding to *attTn7*. The reaction conditions are described in Materials and methods. The DNA substrate (96 bp) used in all TnsD binding reactions contains *attTn7* (+23 to +58), the TnsD binding site. TnsD (6.7 ng) was present in lanes 3 and 4. Crude extract (1.48 μg) was present in lanes 2 and 4. The numbers to the right of the gel represent the mol wts of the gel markers ([^{35}S]DNA mol. wt marker, Amersham). The results are shown are of a scanned gel.

Liebold *et al.*, 1995; Noller and Nomura, 1996); ACP is an essential protein whose major role is in fatty acid biosynthesis as a carrier of fatty acids (reviewed by Magnuson *et al.*, 1993). In this report, we demonstrate that the combination of L29 and ACP can influence Tn*7* transposition *in vitro*, suggesting that the interactions between L29, ACP and TnsD–*attTn7* complexes may contribute to high-frequency insertion of Tn*7* into *attTn7*. We have also found that L29 has a substantial impact on Tn*7* transposition into *attTn7 in vivo*.

Results

Site-specific insertion of Tn*7* into *attTn7* requires the binding of TnsD to this target site. It has been observed previously that host proteins present in crude extracts can stimulate binding of TnsD to *attTn7*, as evaluated by a gel mobility-shift assay, resulting in the formation of a new slower-mobility TnsD–*attTn7* complex (Figure 1, lane 4 versus lane 3) (Bainton *et al.*, 1993). Host proteins do not bind to *attTn7* in the absence of TnsD (Figure 1, lane 2). It should be noted that in all DNA-binding reactions the amount of TnsD is limiting; the stimulation in TnsD–*attTn7* complex formation is more apparent under these assay conditions. We have ruled out the involvement of HU, IHF, H-NS and FIS in the formation of TnsD–*attTn7* complexes; these small heat-stable proteins that have been shown to participate in various recombination reactions do not influence TnsD binding to *attTn7* (data not shown). Thus, another host protein(s) must be involved in altering the formation of TnsD–*attTn7* complexes.

Identification of two host factors that stimulate TnsD binding

To identify the host proteins that influence TnsD–*attTn7* complex formation, we fractionated crude extracts (see Materials and methods) and evaluated fractions for their ability to promote TnsD–*attTn7* complex formation at each purification step using the appearance of altered TnsD–*attTn7* complexes as an assay. Fractionation of cell extracts over a phosphocellulose (PC) column separated the host activity into two components. One component bound to the PC column, PC_{bound}, and contained a host protein(s) that could alter the migration of TnsD–*attTn7* complexes. The TnsD–*attTn7* complexes formed in the presence of the PC_{bound} factor had migration patterns that were equivalent to TnsD–*attTn7* complexes formed in the presence of unfractionated crude extract. In contrast, the other host component, $PC_{flow\ through}$, enhanced TnsD binding to *attTn7* in the presence of the PC_{bound} factor, but could not influence TnsD–*attTn7* complex formation alone.

The PC_{bound} fraction was further fractionated by cation exchange (MonoS) and gel filtration (Superose 12), which resulted in Component 1. We found that Component 1 alone could alter the binding of TnsD to *attTn7*, resulting in the formation of a new slower-migrating TnsD–*attTn7* complex (Figure 2B, lane 3). We also found that the formation of Component 1 + TnsD–*attTn7* complexes could be greatly stimulated when the $PC_{flow\ through}$ component, which was identified as ACP (see below), was present in the *attTn7* binding reactions (Figure 2C, lanes 5–11). Analysis of the Superose 12 fractions by SDS–PAGE and Coomassie Blue staining revealed that a small polypeptide of ~7.0 kDa (Figure 2A) coincided with fractions displaying Component 1 host activities. The identity of the 7.0 kDa protein was determined using amino-terminal sequencing; the first 12 amino acids of this protein were identified as MKAKELREKSVE. A protein homology search found this sequence to be identical to the ribosomal protein L29.

The $PC_{flow\ through}$ factor(s) were further fractionated by anion exchange (MonoQ) and two components, 2A and 2B, were identified that could influence the formation of L29 + TnsD–*attTn7* complexes. Components 2A and 2B were fractionated once more by gel filtration (Superose 12) and active host fractions were evaluated by SDS–PAGE and Coomassie Blue staining. Component 2A was identified as an ~15.0 kDa protein which was found to coincide with fractions that could greatly enhance TnsD binding to *attTn7* in the presence of L29 (Figure 3A, lanes 3–8, and B, lanes 13–18). In contrast, Component 2B was identified as an ~30.0 kDa protein which was found to coincide with fractions that caused the formation of several distinct slower-mobility L29 + TnsD–*attTn7* complexes (Figures 4A, lanes 2–7, and B, lanes 13–18). Note that neither Components 2A nor 2B alone can influence the binding of TnsD to *attTn7* (Figures 3B, lanes 5–11, and 4B, lanes 5–11).

To determine the identity of the proteins present in both Components 2A and 2B, N-terminal sequencing was used. Surprisingly, we obtained identical amino acid sequences (MTIEERVKKIIG) for both Components 2A and 2B, despite their different fractionation profiles and different observed molecular weights. A protein homology search found this protein sequence to be a perfect match to the *Escherichia coli* ACP. Components 2A and 2B were identified as ACP monomers [$(ACP)_1$] and covalent dimers of ACP [$(ACP)_2$], respectively (see below).

In summary, the fractionation of the host-cell extracts

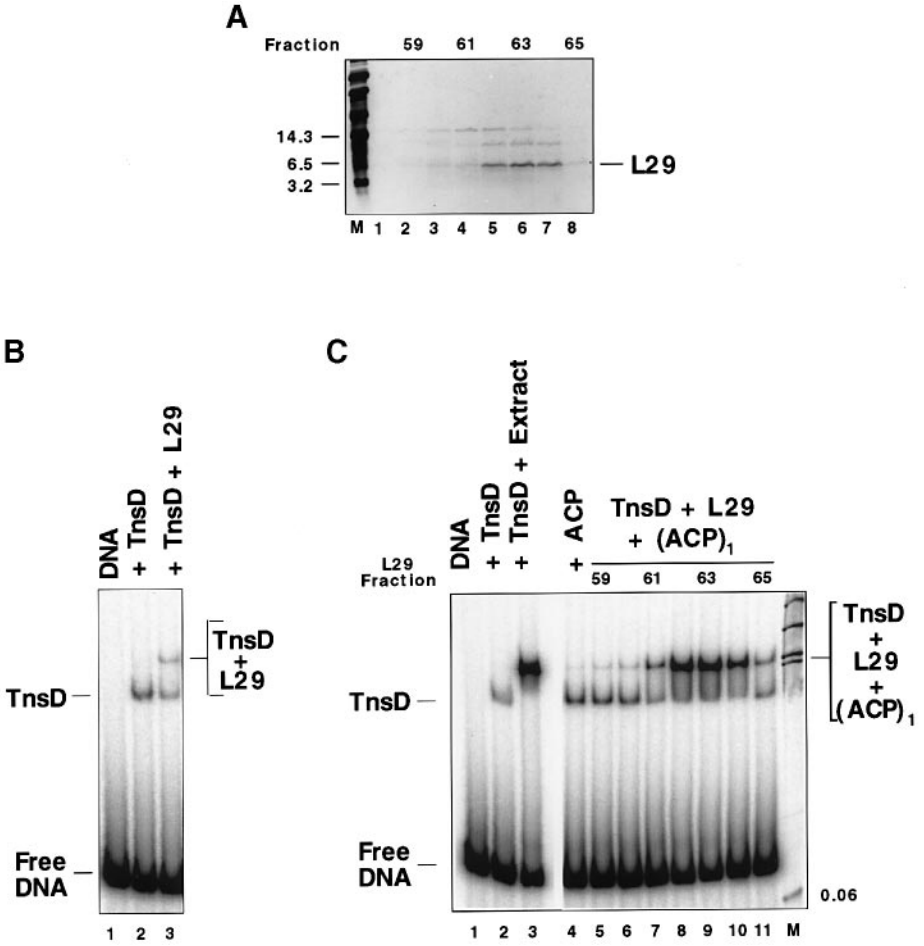

Fig. 2. (**A**) Analysis of L29-containing fractions by SDS–PAGE and Coomassie Blue R250 staining. Lanes 1–8 contain ~30 µl of Superose 12 fractions #58–65, respectively. (**B**) Evaluation of Component 1 (L29) Fraction #64, for its ability to alter binding of TnsD to *attTn7*. TnsD (6.7 ng) was present in lanes 2 and 3, and Fraction #64 (20 ng) was present in lane 3. (**C**) Analysis of L29-containing fractions in the presence of ACP for their ability to alter TnsD binding to *attTn7*. Dilutions of 1:10 of Superose 12 fractions #59–65 (lanes 5–11) were analyzed in each *attTn7*-binding reaction, using the gel mobility-shift assay. TnsD (6.7 ng) was present in lanes 2–11. ACP (15 ng) was present in lanes 4–11. Lane 3 contained ~1.5 µg of wild-type crude extract. The reactions conditions are described in Materials and methods, and the results shown are of a scanned gel.

revealed that L29 causes the formation of slower-migrating TnsD–*attTn7* complexes, and that $(ACP)_1$ and $(ACP)_2$ collaborate with L29 to stimulate binding of TnsD to *attTn7* more than 20-fold compared with the binding of TnsD to *attTn7* in the absence of host factors.

Our purification procedure uses two different *E.coli* strains based on findings in our initial purification attempts. Note that we purified ACP from an L29$^-$ strain which contained a deletion of 48 nucleotides within *rpm*C, the gene encoding L29, to prevent the contamination of ACP with wild-type L29. Since ACP is an essential protein, no ACP null mutants are available: therefore, L29 cannot be purified in the absence of wild-type ACP. Hence, we are unable to exclude the possibility that trace amounts of ACP exist in the L29 preparations.

Two forms of ACP can influence TnsD binding to attTn7

As described above, $(ACP)_1$ (Component 2A) and $(ACP)_2$ (Component 2B) can both alter the formation of L29 + TnsD–*attTn7* complexes. $(ACP)_1$ + L29 enhances the binding of TnsD to *attTn7* >20-fold, causing the formation of a slower-migrating TnsD–*attTn7* complex (Figure 5B, lane 6). In contrast, $(ACP)_2$ + L29 causes the formation of several other distinct slower-migrating TnsD–*attTn7* complexes; the formation of the higher-order complexes suggests the multiple ACP molecules may be capable of interacting with TnsD when bound to *attTn7* (Figure 5B, lane 7). $(ACP)_2$ + L29 + TnsD–*attTn7* complexes can sometimes be detected in the presence of unfractionated extract (data not shown).

$(ACP)_2$ consists of two $(ACP)_1$ molecules linked covalently through disulfide bonds (Rock and Cronan, 1981; reviewed by Magnuson *et al.*, 1993). $(ACP)_2$ can be converted into $(ACP)_1$ in the presence of the reducing agent DTT. To demonstrate whether $(ACP)_2$ was indeed the form of ACP present in Component 2B, we evaluated the effects of $(ACP)_2$ on L29 + TnsD–*attTn7* complexes in the presence and absence of DTT, which promotes the conversion of $(ACP)_2$ into $(ACP)_1$. We observed that when DTT was present in the binding reaction, $(ACP)_2$ resulted in the formation of $(ACP)_2$ + L29 + TnsD–*attTn7* complexes that had migration patterns that were identical to TnsD–*attTn7* complexes formed in the presence of $(ACP)_1$ + L29 (Figure 5B, lanes 6–8). We also found that the migration pattern of the $(ACP)_2$ polypeptide when treated with DTT is the same as that of $(ACP)_1$, as evaluated by SDS–PAGE (Figure 5A, lane 2). Thus, two

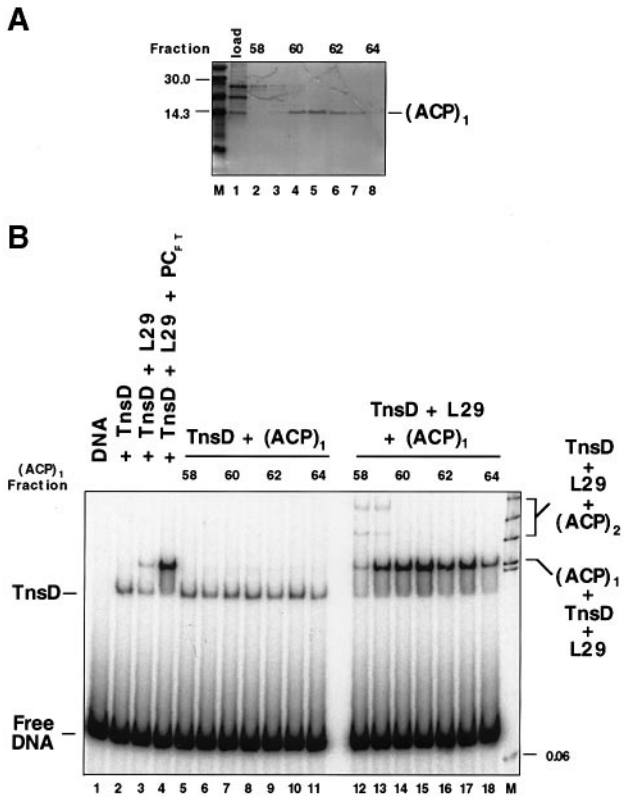

Fig. 3. (**A**) Analysis of $(ACP)_1$ fractions by SDS–PAGE and Coomassie Blue R250 staining. Lanes 1–8 contained ~30 µl of Superose 12 fractions #58–64. (**B**) Analysis of the host activity of the $(ACP)_1$ fractions. Dilutions of 1:10 of Superose 12 fractions #58–64 (lanes 5–18) were analyzed in TnsD binding reactions using the gel mobility-shift assay. Lanes 2–18 contained 6.7 ng of TnsD. L29 (20 ng) is present in lanes 3, 4 and 12–18. Lane 4 contained ~1.5 µg of the MonoQ load (phosphocellulose flow-through). The reaction conditions were the same as those in Figure 1 and are described in Materials and methods. The results shown are of a scanned gel.

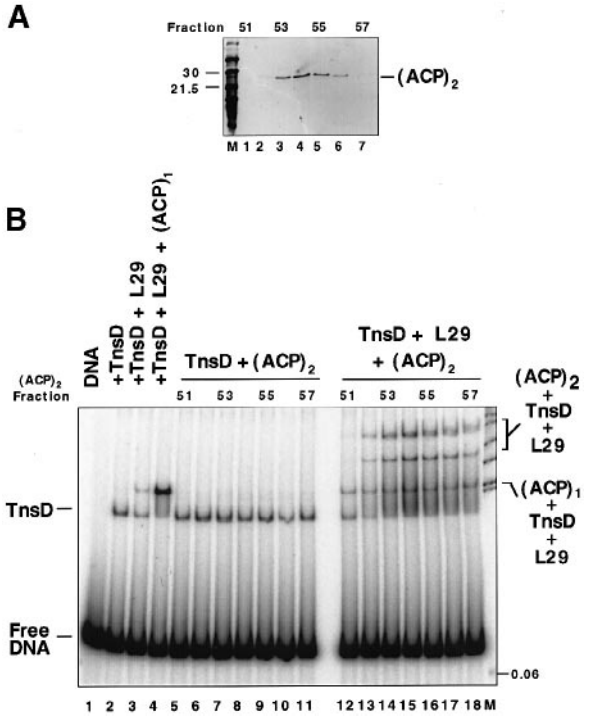

Fig. 4. (**A**) Analysis of $(ACP)_2$ fractions by SDS–PAGE and Coomassie Blue R250 staining. Lanes 1–7 contained ~30µl of Superose 12 fractions #51–57. (**B**) Analysis of the host activity of the $(ACP)_2$ fractions. A 1:10 dilution of Superose 12 fractions #51–57 (lanes 5–18) was analyzed in TnsD binding reactions using the gel mobility-shift assay. TnsD (6.7 ng) was present in lanes 2–18. L29 (20 ng) was present in lanes 2, 3 and 12–18. Lane 4 contained ~1.5 µg of the MonoQ load (phosphocellulose flow-through). The reaction conditions were the same as those in Figure 1 and are described in Materials and methods. The results shown are of a scanned gel.

distinct forms of ACP [$(ACP)_1$ and $(ACP)_2$] can influence the formation of L29 + TnsD–*attTn7* complexes; the ability of $(ACP)_1$ and $(ACP)_2$ to form L29 + TnsD–*attTn7* complexes that have different mobilities on non-denaturing polyacrylamide gels suggests that ACP is a constituent of the slower-migrating TnsD–*attTn7* complexes.

L29 and ACP are present in the TnsD–attTn7-host complexes

We have presented evidence suggesting that both L29 and ACP are involved in stimulating TnsD binding to *attTn7*. Are L29 and ACP present in the TnsD–*attTn7*-host complexes? The fact that purified L29 causes a shift in mobility strongly suggests that it is a component in this complex; ACP, however, causes no additional shift. Therefore, to further examine this question, tritium (^3H)-labeled $(ACP)_1$ was used in the TnsD-binding reactions with unlabeled *attTn7* DNA fragments. The mobility of the TnsD–*attTn7* complexes formed in the presence of both ^3H-$(ACP)_1$ and L29 appeared to migrate identically to the TnsD–*attTn7* complexes formed when [^{32}P]*attTn7* was used to evaluate the effects of unlabeled $(ACP)_1$ on TnsD binding to *attTn7* (Figure 6, lane 15 versus lanes 5 and 10). These data indicate that $(ACP)_1$ is a component of the slower-migrating TnsD–*attTn7* complexes. The ability of the [^3H]$(ACP)_1$ to interact with TnsD–*attTn7* complexes only in the presence of L29 provides additional support for our view that L29 and ACP work together to stimulate binding of TnsD to *attTn7* (Figure 6, lane 15 versus lane 12).

We also detected a novel complex on non-denaturing polyacrylamide gels in *attTn7* binding reactions containing only $L29_{HH}$, a modified version of L29 containing a histidine purification tag and the HSV epitope tag at its C-terminus, and [^3H]$(ACP)_1$, suggesting that these two proteins can interact without TnsD (Figure 6, lane 14). Although *attTn7* DNA is present in the binding reaction, we found that neither L29 nor ACP is able to bind to *attTn7* without TnsD (data not shown). Note that these slower-migrating complexes are not present in binding reactions containing ^{32}P-labeled *attTn7* (Figure 6, lane 9). Thus, it is likely that L29 and ACP can interact through direct protein–protein interactions. In contrast, we have found that interactions between TnsD, L29 and ACP require the presence of the *attTn7* target DNA, suggesting that stimulation in TnsD binding to *attTn7* by L29 and ACP may also involve protein–DNA interactions (data not shown).

To demonstrate that L29 [63 amino acids (aa)] interacts with TnsD–*attTn7* complexes, we evaluated the ability of $L29_{HH}$ (63 + 26 aa) to alter the formation of TnsD–*attTn7* complexes. We found that $L29_{HH}$ + TnsD–*attTn7* complexes migrated more slowly than L29 + TnsD–*attTn7* complexes (Figure 7A, lane 6 versus lane 2). The formation of $L29_{HH}$ + TnsD–*attTn7* complexes of altered

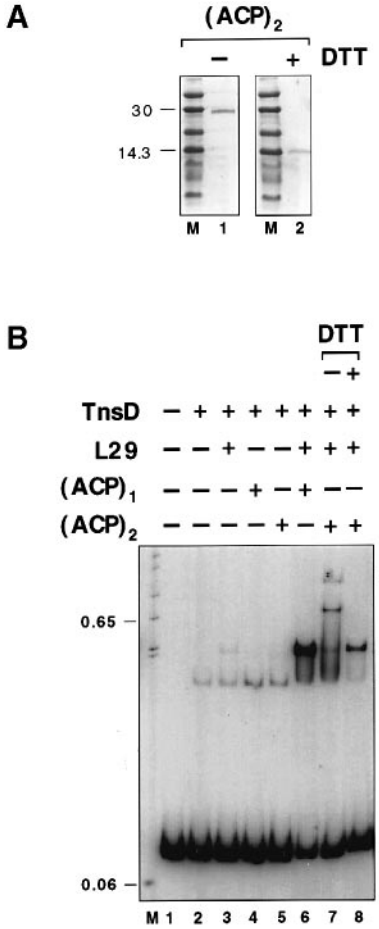

Fig. 5. (**A**) Analysis of $(ACP)_2$ by SDS–PAGE and Coomassie Blue R250 staining ± the reducing agent DTT. (**B**) Analysis of $(ACP)_2$ ± DTT for its ability to influence TnsD binding to *attTn7*. (+) indicates the following protein additions: TnsD (6.7 ng), L29 (20 ng), $(ACP)_1$ (15 ng) and $(ACP)_2$ (8 ng). DTT was added only in lane 8. The reaction and gel conditions were the same as those in Figure 1 and are described in Materials and Methods. The results shown are of a scanned gel.

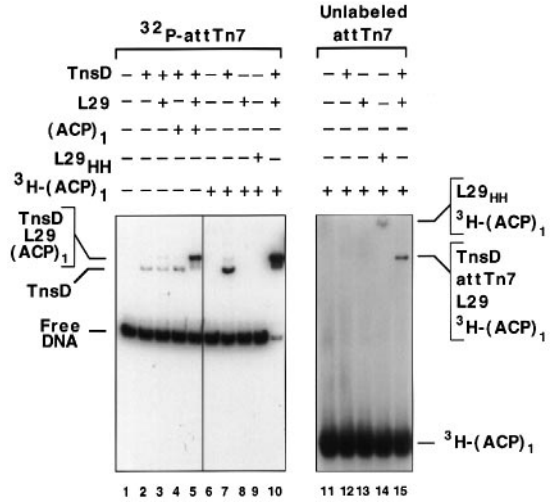

Fig. 6. Evaluation of $[^3H](ACP)_1$ for its ability to influence TnsD binding to *attTn7*. (+) indicates the following protein additions: TnsD 6.7 ng (lanes 2–5) and 40 ng (6–17), L29 20 ng (lanes 3 and 5) and 80 ng (lanes 8, 10, 14 and 16), $(ACP)_1$ (15 ng), $L29_{HH}$ (360 ng) and $[^3H](ACP)_1$ (<1 ng). The DNA substrate (96 bp) used in lanes 1–11 is $[^{32}P]$*attTn7* (+23 to +58). Lanes 12–17 contain unlabeled *attTn7* (+23 to +58). The reaction and gel conditions were essentially the same as those in Figure 1, with one exception: gels were run for 2.5 h instead of 4.0 h. The results shown are of an autoradiograph.

mobility provides evidence that L29 is indeed a component of the TnsD–*attTn7*-host complexes formed in the presence of crude extracts. The formation of $L29_{HH}$ + TnsD–*attTn7* complexes was also stimulated in the presence of $(ACP)_1$ (Figure 7A, lane 7). Note that along with the $L29_{HH}$ + TnsD–*attTn7* complexes, another protein–DNA complex is present in the TnsD-binding reaction that migrates to a position identical to that of L29 + TnsD–*attTn7* complexes. This is likely to result from both $L29_{HH}$ and wild-type L29 being present in the $L29_{HH}$ protein preparation; on Coomassie-Blue-stained SDS–polyacrylamide gels both $L29_{HH}$ and wild-type L29 are visible (data not shown). The presence of wild-type L29 in the $L29_{HH}$ preparation suggests that L29 can form multimers, although we have no evidence indicating that the multimeric state of L29 is critical to its role in Tn*7* transposition.

L29 was further determined to influence TnsD–*attTn7* complex formation by: (i) showing that host extracts made from a strain containing an internal deletion of 16 amino acids within L29, $L29^{\Delta 9\text{-}24}$, could no longer stimulate the formation of TnsD–*attTn7* complexes (Figure 7B, lane 6); (ii) establishing that the combination of purified L29 and the $L29^{\Delta 9\text{-}24}$ extract stimulates TnsD binding to *attTn7* to a level that is indistinguishable from that of unfractionated wild-type host extracts (Figure 7B, lane 7); and (iii) demonstrating that L29 (provided by K.Neirhaus) that was purified from a different purification procedure causes the formation of a protein–DNA complex whose migration pattern is identical to that of L29 + TnsD–*attTn7* complexes that is formed in the presence of our L29 preparation (Figure 7B, lane 5). Together, these observations strongly suggest that L29 is a constituent of TnsD–*attTn7* complexes formed in the presence of crude extract and is indeed involved in stimulating TnsD–*attTn7* complex formation.

L29 and ACP can stimulate Tn7 transposition in vitro

We demonstrated above that L29 and ACP can affect TnsD binding to *attTn7*. Can L29 and ACP also influence Tn*7* transposition? To determine whether L29 and/or ACP can alter Tn*7* transposition *in vitro*, the effects of both host proteins on Tn*7* insertion into an *attTn7*-containing target were evaluated in a reconstituted system using purified Tn*7*-encoded transposition proteins. The Tn*7* transposition *in vitro* reaction is very efficient under standard reaction conditions, and thus does not permit the effects of L29 and ACP on Tn*7* transposition to be adequately evaluated. However, we found that when the amount of TnsD in the transposition reaction is limiting, an effect of L29 and ACP on Tn*7* transposition *in vitro* can be detected; other reaction conditions tested did not have notable effects on the transposition reaction (data not shown). It should be noted that the combination of L29 and ACP causes an increase of at least three-fold (for the 1/3X and 1/9X TnsD dilutions) in Tn*7* insertion into the *attTn7* target site (Figure 8, lanes 7–16). The molar amounts of L29 and ACP present in transposition reactions are similar to TnsD levels; i.e. the amount of L29 and ACP needed to stimulate Tn*7* transposition *in vitro* is not

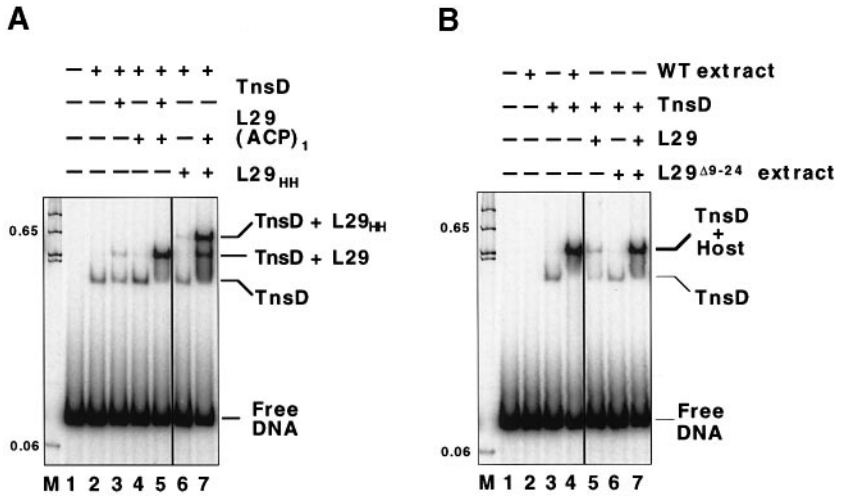

Fig. 7. (**A**) Evaluation of a modified form of L29 ($L29_{HH}$) for its ability to influence TnsD binding to *attTn7*. (+) indicates the following protein additions: TnsD (6.7 ng), L29 (20 ng), $(ACP)_1$ (15 ng) and $L29_{HH}$ (50 ng). The reaction conditions were the same as those in Figure 1 and are described in Materials and methods. The results shown are of a scanned gel. (**B**) Evaluation of the purified L29 and an $L29^{\Delta 9\text{-}24}$ extracts for their ability to influence TnsD binding to *attTn7*. (+) indicates the following protein additions: wild-type cell extract (*E.coli* strain HN1511) (1.48 μg), TnsD (6.7 ng), purified L29 (20 ng) and $L29^{\Delta 9\text{-}24}$ extract (*E.coli* strain AM111–5) (1.56 μg).

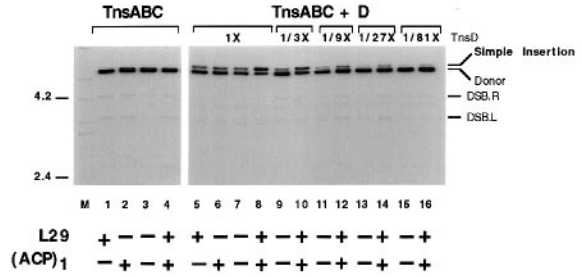

Fig. 8. Analysis of L29 and $(ACP)_1$ on Tn*7* transposition *in vitro*. (+) indicates that L29 and/or ACP was added to the transposition reaction. The effects of L29 and/or ACP are shown by the levels of simple insertion products. Lanes 3, 7, 9, 11, 13 and 15 received only the addition of host buffer, lanes 1 and 5 received only L29, and lanes 2 and 6 received only ACP. Both L29 and ACP were added to lanes 4, 8, 10, 12, 14 and 16. The concentrations of TnsD were as follows: 1× (22 ng), 1/3× (7.3 ng), 1/9× (2.4 ng), 1/27× (0.81 ng) and 1/81× (0.27 ng). The results shown are of an autoradiograph.

vastly different from the concentrations of the transposition proteins. These results indicate that stimulation of TnsD binding to *attTn7* by L29 and ACP results in enhanced Tn*7* transposition. Notably, neither L29 nor ACP alone can alter Tn*7* transposition *in vitro* (Figure 8, lanes 5 and 6 versus lane 7).

L29 plays a role in Tn7 transposition in vivo

We have also examined the effect of L29 on Tn*7* transposition into the chromosomal *attTn7* site *in vivo*. This was accomplished by evaluating the level of Tn*7* insertions into *attTn7* in the wild-type L29 and the $L29^{\Delta 9\text{-}24}$ strains by probing a Southern blot with a DNA probe specific for the *attTn7* target site (data not shown). We found that a considerable fraction (1–3%) of the chromosomal *attTn7* sites of the wild-type L29 strain received an Tn*7* insertion; comparable levels of insertions have been observed for other strains (DeBoy and Craig, 1996). We were unable to detect Tn*7* insertions into the chromosomal *attTn7* site of the $L29^{\Delta 9\text{-}24}$ strain, indicating at least a 9-fold decrease in transposition. However, we found that when the L29 mutation was complemented by supplying the cells with wild-type L29 on a plasmid, the level of transposition into the chromosomal *attTn7* site was restored to that observed in the wild-type L29 strain. This finding indicates that the defect in Tn*7* transposition is due to L29.

We also evaluated the effects of L29 on Tn*7* transposition using an assay that monitors Tn*7* transposition from the chromosomal *attTn7* site into a conjugable plasmid containing the essential *attTn7* sequences (see Materials and methods). In cells containing wild-type L29, Tn*7* transposition into *attTn7* occurs at a high frequency (Table I). Strikingly, in the $L29^{\Delta 9\text{-}24}$ strain, Tn*7* insertion into *attTn7* decreased >100-fold (Table I). We also evalu-

Table I. Effect of L29 on Tn*7* transposition

Donor strain	Genotype	Target plasmid	Transposition frequency[a]
PS169	*rpm*C	pOX38–*attTn7*	2×10^{-2}
PS167	*rpm*C106[b]	pOX38–*attTn7*	9×10^{-5}
PS168	*rpm*C	pOX38–Gen	2×10^{-6}
PS166	*rpm*C106	pOX38–Gen	5×10^{-6}

Mating-out assays were performed using the following donor strains: PS166, PS167, PS168 and PS169. All of the donors strains contain an insertion of Tn*7* in the *attTn7* target site (*attTn7*::Tn*7*) and are resistant to trimethoprim (Tp^R). Strains PS168 and PS169 contain a wild-type allele of *rpm*C, the gene encoding L29, and strains PS166 and PS167 contain the *rpm*C106 allele, which results in a mutant form of L29 that is missing amino acids 9–24. Each donor strain also contained one of two target plasmids pOX38–*attTn7* or pOX–Gen; both plasmid confer resistance to gentamycin (Gn^R). CW51 (Nal^R, Rif^R) was the recipient strain in all mating reactions. Procedures used to evaluate the mating mixtures are described in the Materials and methods. We found that Tn*7* insertion into the chromosomal *attTn7* site in the L29 mutant strain (AM111-5) can be restored to wild-type levels by providing cells with L29 on a plasmid (data not shown).
[a]The transposition frequency is equal to the number of transposition events and was determined by dividing the number of recipient cells obtaining a target plasmid which contained an Tn*7* insertion ($Nal^RRif^RTp^R$ colonies) by the total number of recipient cells obtaining a target plasmid ($Nal^RRif^RGen^R$ colonies).
[b]The *rpm*C106 allele was generated by a spontaneous deletion of 48 nucleotides near the 5′ end of *rpm*C.

ated the effects of an L29 mutation on the non-TnsD Tn*7* transposition pathway that directs Tn*7* insertions to non-*attTn7* target sites. Tn*7* insertion into non-*attTn7* target sites was similar in both the wild-type L29 and the $L29^{\Delta 9\text{-}24}$ strains. These results indicate that L29 plays an important role in Tn*7* transposition into *attTn7* within the cell. We have not yet been able to evaluate the effect of ACP on Tn*7* transposition; since ACP is an essential protein, addressing this question is technically challenging.

These results support the view that L29 has an important role in directing Tn*7* insertion *in vivo* into target DNAs, plasmid or chromosomal, that contain the TnsD binding site, i.e. *attTn7*, but not non-*attTn7* target sites.

Discussion

The transposon-encoded protein TnsD is responsible for directing Tn*7* insertions into the chromosomal site, *attTn7*: TnsD binds specifically to *attTn7* and recruits the rest of the transposition machinery to this target site. In this work, we report the identification of two host-encoded proteins that together stimulate TnsD binding to *attTn7*. We also provide evidence that these host factors stimulate Tn*7* transposition *in vitro*. Interestingly, a host factor(s) also influences Tn*7* transposition *in vivo*.

L29 and ACP can stimulate TnsD binding to attTn7

We have identified two host proteins, L29 and ACP, that collaborate to stimulate the binding of TnsD to *attTn7*. L29 is a relatively small (63 aa) basic protein that binds to 23S ribosomal RNA and is also a component of 50S ribosomal subunits (Bitar, 1975; Urlaub *et al.*, 1995; Wittmann-Liebold *et al.*, 1995; Noller and Nomura, 1996). The function of L29 in protein synthesis is not known; as L29 null mutants have not been characterized, it is not clear whether L29 is essential for cell viability. However, a strain containing an internal deletion of 16 amino acids, $L29^{\Delta 9\text{-}24}$, is viable (Dabbs, 1979). Ribosomal proteins have been implicated in cellular processes such as replication, transcription, RNA processing and DNA repair (reviewed by Wool, 1996). Notably, this work is the first report of a ribosomal protein participating in bacterial transposition.

ACP is a small (77 aa) acidic protein that functions as a carrier of various fatty acids in several biosynthetic pathways, including fatty acid biosynthesis (reviewed by Magnuson *et al.*, 1993), phospholipid biosynthesis (reviewed by Cronan and Rock, 1996), lipopolysaccharide biosynthesis (reviewed by Raetz, 1996) and activation of hemolysin (Issartel *et al.*, 1991). The ACP polypeptide is post-translationally modified through the transfer of 4′-phosphopantetheine (4′-PP) from coenzyme A to Ser36 of ACP, forming $(ACP)_1$. $(ACP)_1$ is further modified at the terminal sulfhydryl moiety by the enzymatic attachment of various fatty acids, which leads to the formation of several different acylated derivatives of $(ACP)_1$. $(ACP)_1$ can also form covalent dimers with itself through the formation of disulfide bonds, forming $(ACP)_2$ (Rock and Cronan, 1981; Magnuson *et al.*, 1993).

We report here that ACP + L29 results in the formation of TnsD–*attTn7* complexes that are similar in their mobilities (on non-denaturing polyacrylamide gels) to the TnsD–*attTn7*-host complexes that are formed in the presence of crude extracts. Since we found that L29 + unmodified ACP (the ACP polypeptide which lacks the 4′PP moiety and was a gift from C.Walsh) can also stimulate TnsD binding to *attTn7*, it seems unlikely that the prosthetic groups of modified forms of ACP play a role in the formation of L29 + TnsD–*attTn7* complexes (data not shown). Thus, it is most likely that a region common to all ACP molecules is responsible for interacting with L29 + TnsD–*attTn7* complexes. It should be noted that we have not directly evaluated acylated derivatives of ACP for their effects on TnsD binding to *attTn7*; therefore, we cannot speculate on the potential role of acyl–ACPs on Tn*7* transposition.

The evidence presented here suggests that both L29 and ACP can associate with TnsD when it is bound to *attTn7*. We found that an altered form of L29 causes the formation of TnsD–*attTn7* complexes that migrate more slowly than L29 + TnsD–*attTn7* complexes, providing evidence that L29 is indeed a component of the TnsD–*attTn7*-host complexes. We have also shown that ACP is a constituent of the TnsD–*attTn7*-host complexes by demonstrating that slower-mobility TnsD–*attTn7* complexes can be visualized in the presence of $[^3H](ACP)_1$ + L29.

L29 and ACP can stimulate Tn7 transposition

Many features of Tn*7* transposition into *attTn7 in vivo* have been realized using an *in vitro* transposition system, a highly efficient reaction requiring only purified TnsABC+D, the mini-Tn*7* element and *attTn7*-containing target DNAs. Prior to this work, there was no obvious role for host proteins in Tn*7* transposition *in vitro*, although a stimulation of Tn*7* transposition *in vitro* has been detected in the presence of host extracts. We have demonstrated here that L29 and ACP can enhance Tn*7* transposition into *attTn7 in vitro* at least three-fold. It should be noted, however, that both L29 and ACP are necessary to detect any increase in the amount of simple insertion products produced; thus, the host activities of L29 and ACP in Tn*7* transposition *in vitro* are dependent upon each other. It is important to note that the *in vitro* transposition assay was developed and optimized without host proteins. Thus, it may be that our standard assay conditions are not conducive for studying the effects of host proteins on Tn*7* transposition *in vitro*; it is possible that more dramatic effects can be observed under other assay conditions.

ACP has also been reported to stimulate nicking at the 3′ ends of Tn*3* by the purified Tn*3* transposase, although it is not clear whether ACP alters the binding of the Tn*3* transposase to the 3′ ends (Maekawa *et al.*, 1996). Here we have been able to show a direct effect of ACP on Tn*7* transposition *in vitro*. Owing to the essential role of ACP *in vivo*, its effects on Tn*7* transposition *in vivo* have not yet been evaluated.

Most interestingly, L29 was found to play a vital role in Tn*7* transposition *in vivo*. Tn*7* transposition into *attTn7* decreases >100-fold when intact L29 is absent from cells. Moreover, L29 specifically affects Tn*7* transposition reactions involving TnsD; this specificity seems reasonable, since L29 was identified based on its role in enhancing TnsD binding to *attTn7*.

L29 and ACP may play a regulatory role in Tn7 transposition

Tn*7* transposition is a tightly regulated process. The selection of appropriate target sites controls the initial

steps of the transposition reaction. TnsD is responsible for selecting the *attTn7* target site by binding to a specific sequence within *attTn7* (Waddell and Craig, 1988; Bainton *et al.*, 1993). It has been previously established by DNA footprinting analysis that TnsD interacts with TnsC, thus recruiting the TnsA+B transposase bound to the Tn*7* ends to *attTn7*. Thus, a nucleoprotein complex, containing TnsABC+D, *attTn7* and the mini-Tn*7* ends, is essential for promotion of Tn*7* transposition into *attTn7*.

In this work, we have demonstrated that the combination of L29 and ACP enhances the formation of TnsD–*attTn7* complexes and stimulates Tn*7* transposition *in vitro*. Moreover, we have observed that L29 is extremely important for high-frequency Tn*7* insertion into *attTn7 in vivo*. Hence, host proteins may indeed play an essential role in Tn*7* transposition within the cell.

By what mechanism might L29 and ACP influence Tn*7* transposition? The fundamental activity of L29 and ACP is to increase the apparent affinity of TnsD for *attTn7*. Perhaps L29 and ACP promote conformational changes within TnsD that allow TnsD to bind more stably to *attTn7*, thereby increasing the frequency of Tn*7* transposition into *attTn7*. It is possible that L29 and ACP directly function as an anchor, securing TnsD to *attTn7* through protein–protein and/or protein–DNA interactions. We have presented some evidence here showing potential protein–protein interactions between L29 and ACP; whether L29 and/or ACP can bind to *attTn7* when bound to TnsD remains to be established.

Why might L29 and ACP be involved in Tn*7* transposition *in vivo*? One attractive hypothesis is that L29 and ACP modulate Tn*7* transposition into *attTn7* by signaling to Tn*7* when cellular conditions are favorable or unfavorable for insertion. An intriguing but unexamined issue is the possibility that the amount of L29 available to participate in Tn*7* transposition may vary during the different cellular growth phases. The transient availability of L29 may serve to modulate Tn*7* insertion into *attTn7*, thereby linking Tn*7* transposition to cellular growth. As a component of the 50S ribosomal subunit, L29 exists in two different forms (free and bound) within cells. We have provided strong evidence in this work that free L29 + ACP has a significant impact on both binding of TnsD to *attTn7* and Tn*7* transposition *in vitro*. Since the majority of L29 is found in the ribosomes during logarithmic growth, it is intriguing to speculate that 50S ribosomal subunits, which contain L29, might also be capable of influencing TnsD binding to *attTn7*. It has been shown in the case of the ribosomal protein S10 (nusE) that both free S10 and S10, present in 30S ribosomal subunits, and 70S ribosomes can participate in transcription antitermination by phage λ N protein (Das *et al.*, 1985).

It is more difficult to speculate about how information about cell growth can be transmitted in a ACP-dependent fashion. ACP is a very abundant protein and is available during all cellular growth phases. We have evidence that the unmodified ACP polypeptide (apo-ACP) can influence TnsD binding to *attTn7*; therefore, it seems unlikely that the different forms of ACP can regulate transposition. Since only the combination of ACP and L29 can stimulate TnsD–*attTn7* complex formation and Tn*7* transposition *in vitro*, it seems reasonable to suggest that ACP might also assist L29 in modulating Tn*7* transposition within the cell. Understanding how L29 and ACP influence Tn*7* transposition may provide insight into how the molecular mechanisms of Tn*7* transposition are linked to cellular metabolism and may also help reveal the critical features of TnsD interactions with *attTn7*.

Materials and methods

Bacterial strains

L29 was identified from crude extract prepared from HN1125 [*E.coli galK*2 *hupA*16::Kan^R *hupB*11::Tet^R *sup*O str^R] (Wada *et al.*, 1988). HN1511 [*E.coli galK*2 *supO* str^R] is the isogenic parent of HN1525 (Bachmann, 1972). ACP and $(ACP)_2$ were identified from crude extract prepared from AM111–5 [*E.coli gdhA*2 *his*-95 *metB*1 *relA*1 *rpmC rna*-19 *spoT*1] (Dabbs, 1979). A19 [*E.coli gdhA*2 *his*-95 *metB*1 *relA*1 *rna*-19 *spoT*1] is the isogenic parent of AM111–5.

Host crude extracts made from the following strains were used to evaluate their effect on TnsD binding to *attTn7*. P1*vir* transduction was used to transfer each mutation into the same strain background (Miller, 1972). The HU$^-$ strain was made by P1 transduction of *hupA*16::Kan^R *hupB*11::Tet^R from HN1525 into HN1511. The FIS$^-$ strain was created by P1 transduction of *fis*767::Kan^R from OH76 into HN1511. The IHF$^-$ strain was made by P1 transduction of *hip*::Cm^R from BD784 and *him*A::Tet^R from BD783 (laboratory strains) into HN1511. The H-NS$^-$ strain was made by transducing HN1511 with a *hns*205::Tet^R P1*vir* lysate (Dersch *et al.*, 1993).

The following strains were used in the mating-out assay. PS164 was made by P1 transduction of *attTn7*::Tn*7* from OH14 (Hughes, PhD. thesis) into a AM111-5 strain background. PS165 was made by P1 transduction of Tn*7* from OH14 into an A19 strain background. Several strains, PS166, PS167, PS168 and PS169, served as donors in the mating-out assay. PS166 consists of PS164 containing pOX38–Gen (a derivative of the conjugable F factor which has gentamycin resistance, Gen^R), and PS167 consists of PS164 containing pOX38–*attTn7* (pOX–Gen containing *attTn7* sequences). PS168 is PS165 containing pOX38–Gen and PS169 PS165 containing pOX38–*attTn7*. CW51 [*E.coli* F$^-$ *ara arg lac pro*XIII *recA*56 nal^R rif^R] was the recipient strain used in all matings (Waddell and Craig, 1988).

TnsD·His was purified from NCM533 [*E.coli* K-12[λ$^+$] *lacZ*::Tn5*lacI*^{q1}] (Bainton *et al.*, 1993). $L29_{HH}$ was purified from BL21(λDE3) (Novagen). Tritium-labeled ACP was prepared from SJ16 [*E.coli* F$^-$ *panD zad*220::Tn*10 metB relA*1 λ^{r}λ$^-$ *gyrA*216] (Jackowski and Rock, 1981).

PCR primers

The following DNA primers were used during the cloning of TnsD·His and $L29_{HH}$ (see below). NLC129, 5′- TTATCTGTTAAGCATAAAGACTGGC-3; NLC186, 5′-CGAGGATCCGCCAAAACAGCCAAGCTTGGCTACAGG-3′; NLC287, 5′-GGGAATTCCATATGAAAGCAAAAAGAGCTGCGTGAGAAGAGCG-3′; NLC288, 5′-CCGCTCGAGACCCGCCTTCTCGTTCAGTAAAGTCTTAACGCG-3′.

Affinity purification of TnsD·His

TnsD·His contains all 508 amino acids of TnsD (with the exception of a change in the second amino acid of Gly to Ser) fused to a 15-amino-acid linker containing a 10-residue C-terminal histidine tag; its activities are indistinguishable from those of authentic TnsD (data not shown). The TnsD expression plasmid, pPLS24, was constructed by inserting the *Nco*I–*Spe*I fragment from pKAO41 (Orle and Craig, 1991) and the *Spe*I–*Bam*HI PCR fragment amplified from pKAO41, which was obtained using PCR primers NLC129 and NLC186, into the *Nco*I–*Bam*HI site of pGD108 (Ghrayeb *et al.*, 1984).

The TnsD·His protein was purified from NCM533 (Shand *et al.*, 1991) containing the pPLS24 *tnsD* histidine tagged plasmid. Cells were grown at 17°C to an OD_{600} = 1.0 in LB medium supplemented with 100 μg/ml carbenicillin, IPTG was added to 100 μM and the cells were allowed to grow for an additional 4 h. The cells were harvested by centrifugation and resuspended at 4°C in Buffer A (60 mM imidazole, 20 mM Tris pH 7.9, 500 mM NaCl) at 0.5 g cells/ml. The cells were then lysed by sonication and centrifuged at 26 000 *g* for 30 min, and the resulting supernatant was filtered through a 0.45 μm syringe filter (Nalgene). The filtrate was applied to Ni^{2+} Sepharose B resin (Pharmacia) in batch and the resin was washed several times in Buffer A containing 10% glycerol. The washed Ni^{2+} resin was then packed into a 10 ml column, which was washed with several column volumes of Buffer A containing 10% glycerol, and Buffer B (100 mM imidazole, 20 mM

Tris pH 7.9, 500 mM NaCl, 10% glycerol). TnsD·His was eluted from the column with Buffer C (200 mM imidazole, 20 mM Tris pH 7.9, 500 mM NaCl, 10% glycerol). Peak fractions were pooled, dialyzed against 500 mM KCl, 50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 2 mM DTT and 25% glycerol, and stored at –80°C. Approximately 25 μg/g of cells were collected; the final TnsD·His preparation was >95% pure.

Purification of L29 and ACP

We identified the ribosomal protein L29 and the ACP by fractionating a crude cell extract from HN1525 and AM111-5, respectively. Cells were grown at 37°C to an OD_{600} = 1.0 in LB medium. All purification steps were performed at 4°C, unless otherwise specified. For each gram of cell pellet, four milliliters of lysis buffer (10 mM potassium phosphate pH 7.0, 1 mM EDTA, 5 mM β-mercaptoethanol (β-ME), 0.1 mM PMSF, 500 mM NaCl) was used to resuspend the cell pellets. The cells were sonicated and the cellular debris was removed by centrifugation at 26 000 *g* for 30 min at 4°C. Polyethyleneimine (PEI) pH 7.9 was added to a final concentration of 0.5% over 10 min, and the mixture was centrifuged for 15 min at 26 000 *g*. The host extract was boiled while stirring for two min and the denatured proteins were removed by centrifugation at 12 000 *g* for 15 min. The remaining proteins were precipitated with ammonium sulfate, which was added to 100% saturation. Lysis buffer was used to resuspend the ammonium sulfate pellet, which was dialyzed overnight in a low-salt buffer (10 mM potassium phosphate pH 7.0, 1 mM EDTA, 5 mM β-ME, 0.1 mM PMSF).

The lysate was then loaded onto a 2.5 ml phosphocellulose column (Whatman P11) and bound proteins were eluted over a 50 ml linear gradient, 75–769 mM potassium phosphate (pH 7.0). Fractions containing host factors that caused the formation of slower-mobility TnsD–*attTn7* complex (active fractions) eluted in ~300 mM potassium phosphate. The active fractions were pooled, dialyzed into 50 mM potassium phosphate, 50 mM NaCl, and 1 mM EDTA, and loaded onto a MonoS HR 5/5 column (Pharmacia). Proteins that bound to the MonoS column were eluted over a 20 ml linear gradient of 50–525 mM NaCl and the host activity was recovered at around 320 mM NaCl. The active fractions were pooled and concentrated using the Centricon-3 centrifugal concentrators (3000 mol. wt cut-off) (Amicon) and further fractionated using a Superose 12 HR 10/20 (Pharmacia) column (25 ml). The proteins were collected (250 ml fractions) from the gel filtration column into a high-salt buffer (20 mM Tris pH 7.0, 1 mM EDTA, 525 mM NaCl). Fractions 58–68 were found to be active and were collectively referred to as Component 1. The identity of the protein that co-purified with the active fraction was determined by N-terminal sequencing.

When purifying $(ACP)_1$ and $(ACP)_2$, the flow-through fractions from the phosphocellulose column were pooled, dialyzed into 20 mM Tris buffer (pH 7.0), 50 mM NaCl and 1 mM EDTA, and loaded onto a l ml MonoQ HR 5/5 column (Pharmacia). The proteins were eluted with a 20 ml 50–525 mM linear gradient. The activity of the fractions from the MonoQ column were evaluated in the presence of Component 1 (L29). Fractions containing $(ACP)_1$ (Component 2) eluted at ~420 mM NaCl, whereas fractions containing $(ACP)_2$ (Component 3) eluted at ~475 mM NaCl. Both sets of fractions were combined into separate pools and concentrated via Centricon-3 centrifugal concentrators (Amicon), and loaded onto a Superose 12 HR 10/30 column; 250 μl fractions were collected into a high-salt buffer (20 mM Tris pH 7.0, 1 mM EDTA, 525 mM NaCl), and active fractions were pooled. Amino-terminal sequencing was used to identify the protein that co-purified with the activity.

Affinity purification of $L29_{HH}$

L29·HSV·His ($L29_{HH}$) contains all 63 amino acids of L29 fused to a 26-amino-acid linker containing a 12-residue herpes simplex virus (HSV) epitope and a six-residue His tag. The gene encoding L29, *rpmC*, was amplified from the chromosome of NLC28 using PCR methodology. For cloning purposes, PCR primers NLC287 and NLC288 were designed, which allow *Xba*I and *Xho*I restriction sites to flank the *rpmC* gene. The L29 expression plasmid, pPLS42, was constructed by inserting the *Xba*I–*Xho*I *rpmC* PCR fragment into the *Xba*I–*Xho*I site of pET-25b (Novagen). The $L29_{HH}$ protein was purified from the *E.coli* strain BL21(λ DE3), which contains a plasmid with the gene encoding L29 (*rpm*C) fused to the HSV and His tags. Cells were grown at 37°C to an OD_{600} between 0.7 and 0.8 in LB medium supplemented with 100 mg/ml of carbenicillin, IPTG was added to 100 μM and growth was allowed to continue for an additional 2.5 h. The cells were harvested by centrifugation and resuspended at 4°C in 60 mM imidazole, 20 mM Tris pH 7.9, 500 mM NaCl. The cells were then lysed by sonication and centrifuged at 26 000 *g* for 30 min, and the resulting cleared lysate was filtered through a 0.45 μm syringe filter. The filtrate was then loaded onto a Ni^{2+} Sepharose B column (Novagen) and the column was washed with the same buffer and with 100 mM imidazole, 20 mM Tris pH 7.9, 500 mM NaCl. The $L29_{HH}$ fraction was eluted with 200 mM imidazole, 20 mM Tris (pH 7.9), 500 mM NaCl, dialyzed overnight into 10 mM sodium phosphate, 1 mM EDTA, 0.1 mM PMSF, 5 mM β-ME, and loaded onto a Mono Q HR 5/5 column. $L29_{HH}$ eluted at ~300 mM NaCl. Wild-type L29, expressed from the chromosomal *rpm*C gene, co-eluted with the $L29_{HH}$.

Preparation of tritium-labeled ACP

^{3}H-labeled ACP was prepared essentially as described by Rock and Cronan (1981), using *E.coli* strain SJ16 ($PanD^-$), a gift from John Cronan.

Gel mobility-shift assay

The effect of host proteins on binding of TnsD to *attTn7* was evaluated using a gel-mobility shift assay as described by Bainton *et al*. (1993). All of the reaction mixtures (20 μl final volume) contained buffer components [50 mM Tris–HCl pH 8.0, 140 mM KCl, 1 mM EDTA, 1.8 mM dithiothreitol (DTT), 9.8% (v/v) glycerol, 340 μg/ml bovine serum albumin (BSA), 17 μg/ml sheared salmon-sperm DNA, ~0.01 pmol of ^{32}P-labeled *attTn7* DNA fragments] plus 6.7 ng TnsD and various amounts of each host fraction or purified protein. In experiments evaluating ^{3}H-labeled ACP, ~0.5 pmol of unlabeled *attTn7* DNA fragments were used. All reactions were incubated for 20 min at room temperature and electrophoresed through 8.75% polyacrylamide gels (29:1 acrylamide:N,N′-methylene-bisacrylamide) in Tris-borate–EDTA (TBE) buffer at 11.5 V/cm for 3–4 h. The gels were dried under a vacuum and exposed to X-ray film or PhosphorImager screen. Gels containing ^{3}H-ACP_1 were treated with the autoradiography enhancer Enlightning (Dupont) prior to drying and exposure to X-ray film.

In vitro transposition assay

The *in vitro* transposition reactions were performed essentially as described by Bainton *et al.* (1993). Reaction mixtures (100 μl volume) contained 0.25 nM pMIM donor plasmid, 2.5 nM pRM2 *attTn7* target plasmid, 25 mM HEPES, 2.0 mM DTT, 2.5 mM Tris pH 8.0, 2.0 mM ATP, 50 mg/ml BSA, 100 μg/ml tRNA 20 nM TnsA–His, 3 nM TnsB–His, 10 nM TnsC, various concentrations of TnsD·His, ~14 nM L29, ~11 nM ACP and 15 mM magnesium acetate (MgAc). The reaction mixtures (96 μl), which included everything except the MgAc, were assembled on ice and allowed to incubate at 30°C for 30 min. MgAc (4.0 μl) was added to 15 mM and the reaction mixtures were incubated for an additional 30 min. Reactions were stopped by phenol/chloroform (1:1 v/v) extraction, the DNA was ethanol precipitated, digested with *Sca*I, and analyzed by agarose gel electrophoresis and Southern hybridization using a DNA probe specific for the mini-Tn*7* element. The blots were examined by autoradiography and PhosphorImager screens (Molecular Dynamics).

Mating-out assay

The effect of L29 on Tn*7* transposition was evaluated using the mating-out assay (Waddell and Craig, 1988). To evaluate the influence of L29 on low-frequency Tn*7* insertion into non-*attTn7* sites, we used as a target a derivative of the conjugable F plasmid, pOX38–Gen. To evaluate the effect of L29 on high-frequency Tn*7* insertion into *attTn7*, we used as a target a derivative of pOX38–Gen containing the *attTn7* DNA sequences, which will be referred to as pOX38–*attTn7*. Insertions into pOX38–Gen and pOX38–*attTn7* were evaluated in both the L29 wild-type ($L29^{wt}$) and the L29 mutant ($L29^{\Delta 9\text{-}24}$) strains. The donor strains contained a chromosomal copy of Tn*7*, which confers resistance to trimethoprim (Tp^R) and either pOX38–Gen or pOX38–*attTn7*, which are resistant to gentamycin (Gen^R). The recipient strain, CW51, was resistant to nalidixic acid (Nal^R) and rifampicin (Rif^R). The total number of recipient cells obtaining an F plasmid was determined by selecting for $Nal^RRif^RGen^R$ colonies. Recipients obtaining F plasmids with Tn*7* insertions were detected by selecting for $Nal^RRif^RTp^R$ colonies. The transposition frequency was calculated by dividing the number of recipient cells that received an F plasmids containing an Tn*7* insertion by the total number of recipient cells that received an F plasmid.

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