Selective Recognition of Pyrimidine Motif Triplexes by a Protein Encoded by the Bacterial Transposon Tn7

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The bacterial transposon Tn7 is distinguished among mobile genetic elements by its targeting abilities. Recently, we reported that Tn7 is able to selectively insert adjacent to triple-helical DNA. The binding of TnsC, a Tn7-encoded protein, to the triplex DNA target leads to the specific transposition of Tn7 adjacent to both inter- and intramolecular pyrimidine motif triplexes. Here, we further probe how Tn7 targets triplex DNA. We report that TnsC discriminates between different types of triplexes, showing binding preference for pyrimidine but not for purine motif intermolecular triplex DNA. The binding preferences of TnsC and the Tn7 insertion profiles were obtained using psoralenated, triplex-forming oligonucleotides annealed to plasmid DNAs. Although the presence of psoralen is not required for targeting nor is it alone able to attract TnsC, we show that the location of psoralen within the pyrimidine motif triplex does alter the position of Tn7 insertion relative to the triplex. Comparison between the triplex-targeting pathway and the highly site-specific targeting pathway mediated by the binding of the Tn7-encoded protein, TnsD, to the unique site attTn7, suggests that similar structural features within each target DNA are recognized by TnsC, leading to site-specific transposition. This work demonstrates that a prokaryotic protein involved in the targeting and regulation of Tn7 translocation, TnsC, can selectively recognize pyrimidine motif triplexes.

Keywords: triple helical DNA; target site selection; transposition; triplex-binding protein; psoralen

Unlike most mobile genetic elements, which show relatively little preference in their location of insertion, Tn7 exercises exceptional control over its selection of a target site. Five proteins encoded by this element enable Tn7 to evaluate and respond to various targeting signals, giving rise to two separate but overlapping targeting pathways (Craig, 1996). TnsA, TnsB, TnsC and TnsD (TnsABC + D) promote transposition into a unique site in the chromosome of many bacteria, called attTn7 (Craig, 1991), while TnsA, TnsB, TnsC and TnsE (TnsABC + E) promote transposition into non-attTn7 sites on conjugating plasmids (Wolkow et al., 1996) and into particular regions of the Escherichia coli chromosome (Peters & Craig, 2000). An additional level of control comes from the fact that Tn7 insertion into DNAs that already contain Tn7 is greatly decreased; such targets are said to be “immune” to further Tn7 transposition events (Hauer & Shapiro, 1984; Arciszewska et al., 1989; Stellwagen & Craig, 1997a). Thus, Tn7 can sense and respond to both positive and negative signals within a potential target DNA.

The site-specific TnsABC + D pathway has been reconstituted in vitro, allowing the roles of each of the Tns proteins to be identified (Bainton et al., 1991, 1993). TnsA and TnsB collaborate to form the transposase, carrying out all of the breakage and joining activities for transposition, while TnsC and TnsD (TnsABC + D) promote transposition into non-attTn7 sites on conjugating plasmids (Wolkow et al., 1996) and into particular regions of the Escherichia coli chromosome (Peters & Craig, 2000). An additional level of control comes from the fact that

Abbreviations used: TFO, triplex-forming oligonucleotide.
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Assembly of the Tns proteins with both the transposon and attTn7 target DNA results in site-specific Tn7 transposition into attTn7 (Bainton et al., 1993). In addition to site-specific insertion, the architecture of the transpososome complex ensures that the structurally asymmetric Tn7 element inserts in a specific orientation into attTn7 (Arciszewska et al., 1989).

TnsC plays a central role in the ability of Tn7 to preferentially insert into particular targets while purposely avoiding others. TnsC regulates transposition by responding to various targeting signals and subsequently activating the transposase. The binding of TnsC to the attTn7-TnsD complex leads to insertion of Tn7 into attTn7, while removal of TnsC from immune targets through interactions with TnsB prevents insertions from occurring (Bainton et al., 1993; Stellwagen & Craig, 1997a). Thus, the presence or absence of TnsC is critical to both the targeting and ultimately the regulation of Tn7 transposition.

In the absence of the targeting proteins, TnsD or TnsE, the core transposition machinery TnsABCWT promotes a very low level of non-sequence-specific Tn7 insertions (Biery et al., 2000). Using TnsABCWT, we found that Tn7 can, however, selectively target triplex DNA (Rao et al., 2000). Triplex-directed targeting results from preferential binding of TnsC to the triplex DNA, suggesting that triple-helical DNA contains a positive targeting signal for Tn7 transposition (Rao et al., 2000). A gain-of-function mutant of TnsCWT, TnsCA225V (Stellwagen & Craig, 1997b), which provides a much higher frequency of transposition for TnsABCWT (Stellwagen & Craig, 1997b; Stellwagen, 2001), also promotes insertion adjacent to triple-helical DNA structures (Rao et al., 2000).

A direct comparison of Tn7 transposition adjacent to either a pyrimidine motif triplex DNA or the attTn7-TnsD complex in vitro, revealed strikingly similar patterns of insertion in terms of both the location and orientation of Tn7 insertion into each respective target DNA (Rao et al., 2000; Kuduvalli et al., 2001). These similarities suggest that triplex DNA and the attTn7-TnsD complex contain similar signals and are thus being recognized by TnsC in a similar fashion. However, many questions regarding the unexpected recognition of triplex DNA remained. Are all triplexes targeted by Tn7? What specific structural features of triplex DNA influence recognition by TnsC? How does recognition of triplex DNA by TnsC compare with other triplex-binding proteins?

In this work, we have further probed the mechanism by which Tn7 recognizes triplex DNA. We characterize the triplex-binding abilities of TnsC, and compare it to the five previously reported triplex-binding proteins (Kiyama & Camerini-Otero, 1991; Guieysse et al., 1997; Jiménez-García et al., 1998; Musso et al., 1998; Nelson et al., 2000), all of which are found in eukaryotes (see Table 1). The affinity of these proteins for triplex DNA varies between purine and pyrimidine motifs, with a strong preference for one motif over the other. We demonstrate here that TnsC is able to selectively bind pyrimidine, but not purine motif triplexes, leading to pyrimidine triplex-specific transposition.

The triplex targets used in our assays were constructed using triplex-forming oligonucleotides (TFOs) conjugated to a psoralen intercalator to increase triplex stability under in vitro transposition reaction conditions. Interestingly, we find that altering the position of psoralen within the pyrimidine motif triplex targets, in turn alters the insertion profiles generated by Tn7. This work both provides a deeper understanding of the targeting capabilities of Tn7 and illustrates the critical role of TnsC in the regulation of transposition by sensing and responding to specific signals presented by the target DNA.

**Construction of purine and pyrimidine motif triplex targets**

A variety of plasmid DNA targets with short regions (15 bp) of triplex DNA were generated by using TFOs synthesized with psoralen on either the 5' or 3' end, and UV-induced crosslinking of the TFO to the plasmid (Figure 1). The preferred sequence of psoralen intercalation into duplex DNA, TA, was cloned into both the 3' and 5' ends of the triplex targets to allow interstrand crosslinking to these substrates, and increase triplex stability. Triplex DNA can be formed through the pairing of single-stranded DNA to the major groove of duplex DNA through either Hoogsteen or reversed-Hoogsteen hydrogen bonding, giving rise to the pyrimidine and purine motifs (Soyfer & Potaman, 1996). We constructed both motifs for our in vitro transposition targeting assays. The polypurine tract within the pDUP plasmid is identical for each of the targets, thus allowing direct comparison of the various triplex constructs within our transposition assay. The pH-dependence of pyrimidine motif triplex formation, which arises from the requirement of protonation of cytosine for Hoogsteen hydrogen bonding, is partially negated by the use of nucleoside analogs, such as 5-methylcytosine (Lee et al., 1984; Povsic & Dervan, 1989) and 8-oxo-adenine (Miller et al., 1996). The use of psoralen and of modified bases within our TFOs increased the stability of our triplex targets. While these changes do not alter the polarity of triplex formation (Figure 1), they do stabilize triplexes under in vitro transposition reaction conditions.

**Tn7 targets pyrimidine but not purine motif triplexes**

The targeting preferences of Tn7 for various triplex structures were obtained using TnsABC in vitro transposition reactions. For ease of visualization and quantification of reaction products, the gain-of-function allele of TnsCWT, TnsCA225V, was primarily used with TnsAB, though similar targeting preferences are found for TnsCWT. To
obtain a high-resolution view of the positions of Tn7 insertion within the various targets, products of the in vitro transposition reactions were isolated individually by transformation into E. coli, and sequenced. The insertion profile of 100 sequenced insertions into the parental duplex plasmid, pDup, was compared to the insertion profile for p5PS-Y and p5PS-R (Figure 2(a)). The p5PS-Y target plasmid containing a pyrimidine motif triplex generated both site-specific and orientation-specific insertions of Tn7, with a hot-spot located 23 bp from the 5' triplex-duplex junction. This insertion profile is in good accord- ance with the results of previous studies using similar pyrimidine triplex targets (Rao et al., 2000). By contrast, the profile obtained for the purine motif triplex target, p5PS-R, reveals a “random” distribution of Tn7 insertions, giving a profile similar to that of pDup (Figure 2(a)). In comparison to the profiles generated with the pyrimidine motif triplex targets, the purine triplexes showed neither specific localization nor orientation bias of Tn7 insertion. Furthermore, we found no instance of a double-insertion event, where Tn7 inserted into the same triplex target more than once. This confirmed that target site immunity is maintained for triplex targets.

TnsC binds pyrimidine but not purine motif triplexes

TnsC preferentially binds pyrimidine motif triplexes, leading to recruitment of transposase and subsequent triplex-specific insertions of Tn7 (Rao et al., 2000). We tested if TnsC was able to bind
Figure 2 (legend opposite)
purine motif triplexes, even though Tn7 does not preferentially transpose adjacent to purine triplexes. The affinity of TnsC for pyrimidine and purine motif triplexes was determined by binding assays (Figure 2(b)) that used restriction fragments containing the triplex region from each of the triplex substrates (Figure 1). As the concentration of TnsC increased, the binding of TnsC to only the pyrimidine motif triplexes, p5PS-Y and p3PS-Y, increased over that of the duplex control (Figure 2(b)). TnsC was therefore able to distinguish between purine and pyrimidine triplex targets, by binding preferentially to only the pyrimidine substrates.

The influence of psoralen on the point of insertion

Triplex targets can be constructed using TFOs that contain psoralen conjugated to either the 5’ or the 3’ end. Though previous work using intramolecular triplex targets confirmed that psoralen is not required for triplex targeting, and that targets containing psoralen alone are incapable of invoking specific targeting by Tn7 (Rao et al., 2000), the possibility remained that psoralen could affect the position of Tn7 insertion. This was tested by a comparison of the insertion profiles of p5PS-Y and p3PS-Y triplexes, which contain psoralen on opposite ends of the TFO (Figure 1). While Southern blot analysis demonstrates that both give rise to site-specific transposition adjacent to the triplex region (data not shown), targeting differences were found after sequencing a population of insertions into each target (Figure 3). Though insertion by Tn7 occurred into each target with similar specificity (~75% within a 40 bp region of the triplex) and orientation bias (98% had the right end of Tn7 closest to the triplex), the exact position of Tn7 insertion with regard to the triplex varied (Figure 3). The hot-spot of insertion for the p5PS-Y triplex was found 23 bp from the 5’ triplex-duplex junction, whereas the hot-spot for the p3PS-Y triplex was only 10 bp from this junction (Figure 3). However, in both cases, the hot-spot was a distance of ~25 bp with regard to the site of psoralen intercalation for both triplexes. Thus, we see a strong correlation between the precise position of insertion and the location of the psoralen within the pyrimidine motif triplex.

TnsC recognizes triplexes crosslinked at both ends of the TFO

As the TFOs were covalently attached to only one end of the duplex-binding site, our triplex substrates were theoretically capable of fluctuating between an annealed and single-stranded state, or “breathing”. Thus, one possible explanation for binding and transposition adjacent to triplex DNA was that TnsC was recognizing a single-stranded portion of the triplex target. To probe this model directly, TFOs containing psoralen on both the 5’ and 3’ ends (p5 + 3PS-Y, p5 + 3PS-R) (Figure 1) were tested for both targeting by Tn7 (Figure 4(a)) and binding by TnsC (Figure 4(b)). These constructs eliminate any potential “breathing” by the TFOs at the triplex-duplex junction by covalent intercalation for both triplexes. Thus, we see a strong correlation between the precise position of insertion and the location of the psoralen within the pyrimidine motif triplex.
attachment of both ends of the TFO to the target plasmid through psoralen cross-links. Southern analysis illustrates Tn\textsuperscript{7} inserted specifically into p5\textsuperscript{‡}3PS-Y, while the purine motif triplex, p5\textsuperscript{‡}3PS-R, showed no specific targeting (Figure 4(a)).

Though Southern blot analysis confirms that pyrimidine motif triplexes crosslinked at both ends of the TFO are targeted by Tn\textsuperscript{7}, insertion profiles at the sequence level could not be determined for these targets, as insertion products were not recovered after transformation into \textit{E. coli} (presumably due to an inability to effectively process the two sets of intrastrand crosslink adducts generated by the di-psoralenated triplex).

In terms of binding of TnsC, the affinity of TnsC for p5 + 3PS-Y was consistent with the affinities for TFOs with a single psoralen: TnsC preferentially bound p5 + 3PS-Y but not the p5 + 3PS-R triplex (Figure 4(b)). Tn\textsuperscript{7} was able to bind and target triplexes crosslinked at both ends with the same efficiency as triplexes crosslinked at only one end, suggesting strongly that TnsC is not binding a single-stranded form of the triplex.

We have shown here that Tn\textsuperscript{7} is able to distinguish between purine and pyrimidine motif triplexes as potential targets for site-specific transposition. While TnsC selectively binds pyrimidine motif triplexes, leading to triplex-specific transposition, purine motif triplexes are not bound by TnsC, and Tn\textsuperscript{7} shows no preferential targeting to these structures. The presence of psoralen within a pyrimidine motif triplex is able to modify the insertion profile of Tn\textsuperscript{7}.

Alterations in target DNA structure may provide the effector signal for Tn\textsuperscript{7} transposition

How does TnsC distinguish between purine and pyrimidine motif triplexes? A better understanding of triplex recognition may come from the wild-type Tn\textsuperscript{7} targeting pathway mediated by TnsD. TnsD is a sequence-specific, DNA-binding protein that binds a unique site in the bacterial chromosome called \textit{attTn7}. Formation of the TnsD-\textit{attTn7} target complex results in the site-specific and orientation-specific insertion of Tn\textsuperscript{7} adjacent to the TnsD binding site within \textit{attTn7}. Recent studies have revealed that the binding of TnsD to the major groove of \textit{attTn7} results in asymmetric structural changes in and around its binding site, and that TnsC likely binds the altered conformation of the \textit{attTn7}-TnsD complex in the minor-groove (Kuduvalli et al., 2001). The ability of TnsC to recognize and direct site-specific and orientation-specific insertions adjacent to both the \textit{attTn7}-TnsD complex and triplex DNA raises the possibility that the these two targets contain a similar effector signal for site-specific targeting.

Detailed structural information on pyrimidine motif intermolecular triplexes from molecular modeling, footprinting, NMR studies, and a recent crystal structure (Chomilier et al., 1992; Stonehouse & Fox, 1994; Asensio, 1998; Rhee et al., 1999), reveal that TFOs generate modest changes in the confor-
mation of the duplex target to which they anneal. Specifically, the docking of the TFO splits the major groove, in effect creating two additional minor grooves and, in turn, narrows the minor groove of the duplex. Also, the triplex-duplex junctions are differentially unwound, giving rise to asymmetric distortions. Footprinting studies reveal that the triplex targets used in these studies also contain alterations at the triplex-duplex junctions (Rao et al., 2000; our unpublished results). An attractive hypothesis is that the unique combination of structural features within pyrimidine motif triplexes provokes the binding of TnsC. These features may be similar to the attTn7-TrnsD complex, while purine motif triplexes simply do not contain the specific structural signals required to recruit TnsC. Though few structural studies on purine motif intermolecular triplexes have been done, obvious structural differences may arise due to the opposite orientation of the third strand, for example, which could, in turn, alter the triplex-duplex junctions and groove widths. The fact that TnsC recognizes pyrimidine and not purine motif triplexes rules out the notion that TnsC is simply pausing at a triplex-based molecular “road-block”. Our results confirm that TnsC is not simply responding to a global feature of the pyrimidine triplex target, but to a more subtle signal contained therein.

Figure 4. Binding and targeting assays for triplexes containing psoralen at both the 5’ and 3’ ends. Assay for Tn7 target specificity for the triplexes containing di-psoralenated TFOs (p5 + 3PS-R and p5 + 3PS-Y). (a) Comparison of in vitro transposition reaction products as visualized by Southern blot. The p5 + 3PS-R target shows a ladder of bands corresponding to a population of “random” Tn7 insertions (lane 1) while the p5 + 3PS-Y target generates an accumulation of bands indicating triplex-specific Tn7 insertions (lane 2). The band denoted excised linear transposon (ELT) is an intermediate of the cut and paste transposition reaction. The mini-Tn7 donor substrate (mTn7) and the target DNA are indicated. Each 100 μl reaction had 0.37 pmol of target plasmid DNA, 0.04 pmol of mTn7-containing donor plasmid DNA, 1.2 pmol of TnsA, 0.3 pmol of TnsB, 1.0 pmol of TnsC, 25.9 mM Hepes (pH 7.6), 2.5 mM Tris (pH 7.6), 60 μg/ml BSA, 2.0 mM ATP, 2.0 mM DTT, 5.0 mM KCl, 14.3 mM NaCl, 16 μM EDTA, 120 μM MgCl2, 120 μM Chaps, 4 μM PMSF, 0.4 % glycerol, and 15 mM magnesium acetate. The target plasmid DNA and TnsC-A225V were first mixed and assembled at 30 °C for 20 minutes, and then the remaining components were added and incubated at 30 °C for another 30 minutes. The reaction was stopped with phenol/chloroform-extraction and the DNA precipitated, digested with HindIII, and separated on a 0.6 % agarose gel. The gel was subject to Southern blotting and probed with radiolabeled oligonucleotide (NLC94), corresponding to the right end of Tn7. Following phosphorimaging (Molecular Dynamics), the products were quantified using ImageQuant software (Molecular Dynamics). All of the components except TnsA, TnsB, donor plasmid and magnesium acetate were incubated at 30 °C for 20 minutes to allow target assembly prior to the reaction. The remaining components were added and allowed to react at 30 °C for 20 minutes. Phenol/chloroform-extraction and ethanol-precipitation were done before restriction digest and Southern blot. For ease of visualization and quantification of reaction products, the gain-of-function allele of TnsC, TnsC-A225V, was primarily used; however, similar targeting preferences are found for TnsCwt. (b) The relative affinities of TnsC-A225V for the triplexes containing di-psoralenated TFOs (p5 + 3PS-R and p5 + 3PS-Y) are compared.
Effected of psoralen on Tn7 insertion

The effect of the position of psoralen on targeting further supports the idea that TnsC responds to a combination of particular features within a potential target. The insertion of Tn7 into various pyrimidine triplex targets maintained a distance of ~25 bp from the site of psoralen intercalation. Though purine motif triplexes also contained psoralen, Tn7 did not insert adjacent to these targets. It has been shown that psoralen induces conformational changes within the DNA into which it has intercalated (Hwang et al., 1996). However, without high-resolution structural information on a psoralenated triplex-TnsC complex, it is difficult to understand exactly how the location of psoralen within the pyrimidine motif triplex affects the insertion profile of Tn7. We suspect that the intercalated psoralen moiety mimics a specific aspect of the attTn7-TnsD signal involved in the precise alignment of the transpososome. By moving psoralen from the 5' to the 3' end of the triplex, the alignment of the transposase, and thus the point of Tn7 insertion, is in turn moved a similar distance.

Notable features of the triplex targeting pathway: orientation bias and immunity

The TnsC-triplex interaction leads to Tn7 insertions into a specific location and with a specific orientation. Like the TnsABC + D pathway, Tn7 inserts >98% with a single orientation adjacent to the triplex target (Rao et al., 2000). The similar orientation bias that is found within the triplex pathway reveals that some aspect of the TnsABC machinery, and not TnsD, is responsible for determining the orientation of Tn7 upon insertion.

We also note that double insertions are not observed for either the triplex targets used in this study or for targets that contained two distinct triplex regions on the same plasmid (data not shown). Thus, triplex targets that contain a copy of Tn7 are recognized as immune targets. Like the TnsD-attTn7 target complex, pyrimidine motif triplex DNA represents a positive targeting signal for Tn7 transposition, with the ability to orient and direct the insertion of Tn7 into a specific location.

Implications of the interaction of TnsC with triplex DNA

The identification of proteins that specifically interact with triplex DNA has been used to support the idea that triplex DNA may exist in vivo and may, in fact, play a biological role (Frank-Kamenetski & Mirkin, 1995). Multiple lines of evidence have implicated triplex DNA in several cellular processes, including transcription, replication, and recombination (Soyfer & Potaman, 1996). However, beyond simple binding to triplex DNA, the biological purpose for all previously reported triplex-binding proteins is not known (Table 1). In contrast, the biological role of TnsC is well defined. TnsC regulates Tn7 transposition through interaction with target DNA for the selection of a proper target site, leading to activation of the transposase. A unique feature of the recognition of triplex by TnsC DNA is that TnsC is part of the repertoire of proteins that carry out transposition of Tn7: the binding of TnsC leads to a highly specific transposition event and thus provides a functional assay for triplex formation. We have shown here that our assay provides insight into both the mechanism of target site selection by Tn7 and the potential structural differences amongst

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The binding preferences of the reported triplex-binding proteins for each type of triple-helical motif are listed. A check (tick) mark indicates a preferential binding of triplex over duplex DNA, while (-) implies that the binding was equal to that of duplex controls. An X indicates no observable binding to the motif listed, while the nt indicates that the motif was not tested. The biological roles for each protein are listed. Ref. 1, Rao et al. (2000); 2, Nelson et al. (2000); 3, Jiménez-García et al. (1998); 4, Musso et al. (1998); 5, Guieysse et al. (1997); 6, Kiyama & Camerini-Otero (1991).
purine and pyrimidine triplex motifs. The ability of Tn7 to target triplex regions of DNA for site-specific insertion could be extended to in vivo applications, providing a functional assay for capturing or “tagging” naturally occurring triplex structures such as H-DNA. As direct proof of triplex formation in vitro is lacking, Tn7 may provide a powerful method of characterizing such structures.

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