Nucleic Acids Research, Vol. 18, No. 4

TcA, the putative transposase of the *C.elegans* Tc1 transposon, has an N-terminal DNA binding domain

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Received November 2, 1989; Revised and Accepted January 18, 1990

ABSTRACT

Tc1 is a transposon present in several copies in the genome of all natural isolates of the nematode *C.elegans*; it is actively transposing in many strains. In those strains Tc1 insertion is the main cause of spontaneous mutations. The transposon contains one large ORF that we call TcA; we assume that the TcA protein is the transposase of Tc1. We expressed TcA in *E.coli*, purified the protein and showed that it has a strong affinity for DNA (both single stranded and double stranded). A fusion protein of β-galactosidase and TcA also exhibits DNA binding; deletion derivatives of this fusion protein were tested for DNA binding. A deletion of 39 amino acids at the N-terminal region of TcA abolishes the DNA binding, whereas a deletion of 108 C-terminal amino acids does not affect DNA binding. This shows that the DNA binding domain of TcA is near the N-terminal region. The DNA binding capacity of TcA supports the assumption that TcA is a transposase of Tc1.

INTRODUCTION

Insertion of the Tc1 transposon is the most frequent cause of mutation in the nematode *C.elegans*, at least in those strains in which Tc1 is active[1,2]. All isolates of *C.elegans* contain several copies of the Tc1 element[3] but the element is active in transposition only in some strains[2]. Genetic analysis has shown that a single genetic element present in an active strain but absent in an inactive strain can be responsible for activation of Tc1 transposition; this is possibly a mobile element itself (designated *mut*[4]).

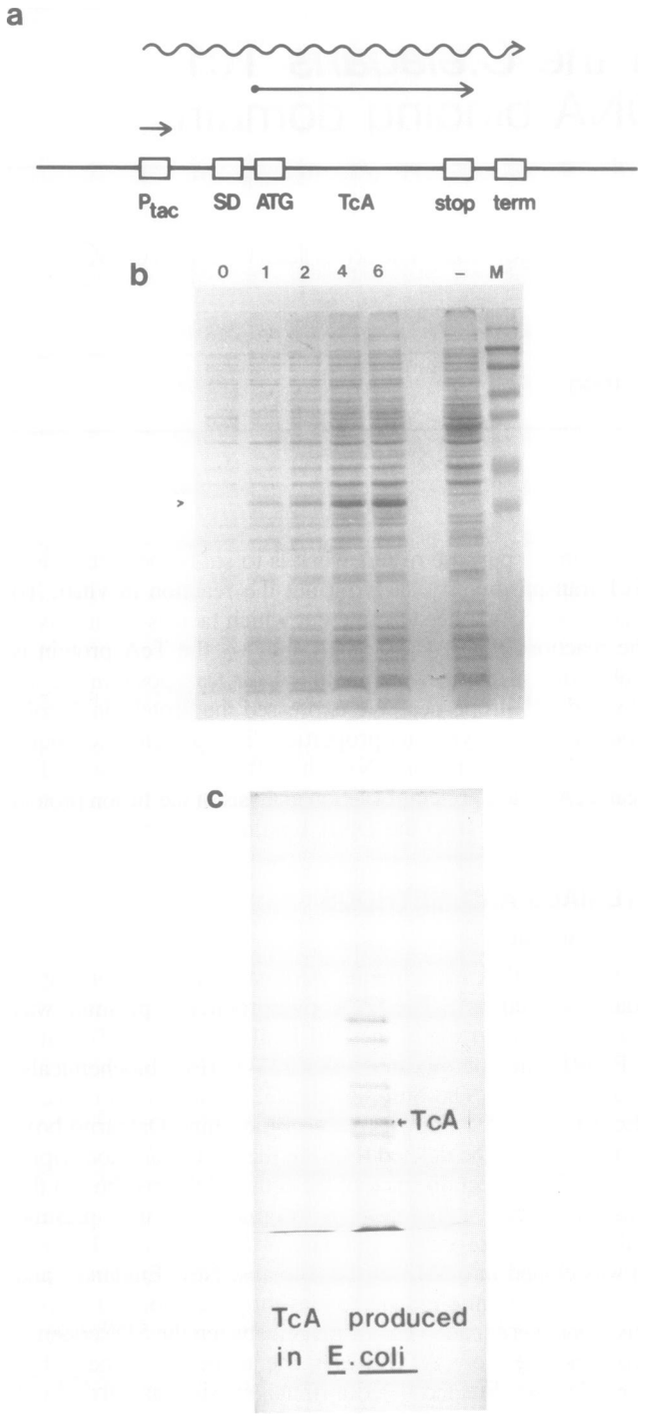
Tc1 is an element of 1612 bp length, with short inverted repeats and one large internal ORF[5]. Although there is some variation in the exact DNA sequence[5,6,7] all Tc1 elements have the same structure; no Tc1-like elements of different size have been detected[4]. The 273 triplet open reading frame in Tc1 largely fills the transposon; we called this ORF TcA. Only one element containing a shortened version of TcA has been described[6]. TcA is strongly conserved between species: it is found in another nematode species[7] and even in *Drosophila*[7,8]. It would not be expected that the TcA gene would be so strongly conserved if it were not essential for the transposon. Therefore it seems likely that the TcA gene product plays a role in transposition for instance as the transposase of Tc1.

The ultimate purpose of our work is to study the mechanism of Tc1 transposition by carrying out the reaction in vitro. To do this we shall first need to find out which factors are involved in the reaction. Since —as argued above— the TcA protein is probably one of the proteins essential for transposition we set out to study its properties. We expressed the protein in *E.coli*, purified it and analyzed its properties. The protein was found to have a high affinity for DNA; this affinity was preserved in a β-gal/TcA fusion protein. Deletion analysis of the fusion protein was carried out to map the DNA binding domain.

MATERIALS AND METHODS

DNA techniques

Recombinant DNA experiments were carried out according to standard techniques[9]. The TcA overproducing plasmid was constructed by insertion of the StuI-DraI fragment of Tc1[5] into the EcoRI site of plasmid pKK223-3 (PL biochemicals, Milwaukee USA). The StuI site cuts 32 bp upstream of TcA, the EcoRI site is 5 bp downstream of the Shine-Dalgarno box. Thus DNA had to be deleted to bring the ATG initiation triplet of TcA at the correct distance of the Shine-Dalgarno box in the expression vector. Therefore a fragment of this plasmid containing the tac-promoter and part of the TcA gene (BamHI-SalI) was cloned into M13 mp18 (Biolabs, New England), and using three different oligonucleotides three deletion derivatives of this clone were made. The distance between the SD sequence AGGA and the TcA ATG was made to be 6, 7 and 8 bp. Oligonucleotide directed deletion mutagenesis was carried out following the protocol of Kunkel et al.[10]. The sequence of the deletion derivatives was checked. All three derivatives were reinserted into the expression plasmid and tested for TcA expression in *E.coli* JM101 after IPTG induction. The clone with the shortest distance between the SD and initiation triplet was found to produce the most TcA. The sequence at the translation start of the TcA gene in the overproducing clone used in this study is shown in the legends to figure 1. Since the initial TcA overproducer was found to produce an unexpectedly small protein, the TcA gene in this clone (derived from the isolate *st137*, provided by Dr.D.Moerman) was sequenced. It was found to contain one amber mutation not present in the previously sequenced isolate of Tc1[5,6]. This region of the clone was replaced by the corresponding sequence of TcA from clone pCe(Be)Tc1 (provided by Dr. S. Emmons, New York), and now



1. TcA expressed in *E.coli*
(a) A schematic representation is shown of the plasmid pRP67. This is a derivative of the expression plasmid pKK223-3 (PL-Biochemicals) that has the TcA gene (StuI-DraI fragment of Tc1) cloned behind the tac promoter and SD sequence. Using an oligonucleotide a deletion was made to bring the ATG initiation triplet of TcA at the correct distance of the SD sequence (see methods section for details). The sequence around the initiation of TcA is CACA AGGA AACAGA ATG AAT CGC; the SD sequence needed for efficient translation initiation in *E.coli*, and the initiation triplet of TcA are underlined. (b) This plasmid was transformed into the strain JM101, and the strain was induced for the indicated time; the arrow shows a protein of 30kD that shows up after the induction. The control lane shows a strain of JM101 that does not contain pRP67 after 6h of induction. (c) From an induced culture of JM101+pRP67 TcA was isolated, and purified as described in the text. The left lane shows purified TcA protein, the right lane the crude extract. After SDS-PAGE the gel was stained with Coomassie Brilliant Blue. The arrow indicates TcA, which we estimate to be over 80% pure.

a full size TcA protein was observed after induction of the resulting clone pRP67.

Construction of TcA-β-gal fusion genes and induction of their expression was as described in Ruether and Mueller-Hill[11]. DNA for 'South-Western blot' probes was radiolabeled using the random priming method (kit purchased from Boehringer Mannheim).

Protein techniques

TcA was purified as follows: 4h after IPTG addition to an exponentially growing JM101+pRP67 strain the cells were collected by centrifugation, and resuspended in 1M NaCl, 2mM DTT, 5mM HEPES pH7.5. The cells were lysed by sonication, and the non soluble fraction was collected by centrifugation (10′ in an Eppendorf centrifuge at maximum speed). The pellet was resuspended in 1M NaCl, 2mM DTT, 5mM HEPES pH7.5, 0.1% Nonidet P40 by repeated pipetting. This washing was repeated twice. Then the pellet was resuspended in the same buffer containing 8M Guanidine-HCl. The insoluble material was again removed by centrifugation in an eppendorf centrifuge at maximum speed (10′). Immuno-blot analysis of pellet and supernatant fractions both of the first washes and of the final wash showed that the TcA protein remained in the insoluble fractions until it was dissolved in 8M Guanidine (not shown). Then the cleared supernatant was dialyzed several hours against the same buffer but now containing 8M urea instead of Guanidine-HCl. Dialysis was continued each time 2h against lower concentrations of urea (4M, 1M, and finally 0M). During this time the protein has time to refold, possibly back into its native conformation. Some precipitate was found in the dialysis tube, but most of the TcA remained in solution after this procedure. TcA protein was now approximately 80% pure (see figure 1c). It was stored at −70C in the presence of 10% glycerol.

Generation of antibodies

A peptide N-Cys-Gln-Val-Ala-Ile-Asp-Ala-Gly-Tyr-Ala-Thr-Lys-Tyr-C (corresponding to the 12 C-terminal amino acids of TcA) was synthesized and coupled to KLH (Keyhole Limpet Hemocyanin, Sigma) by the method described by Green et al.[12]. Rabbits were injected with the antigen as in Green et al.[12]; after the third booster serum was collected.

Protein blots

Proteins were separated on 12.5% SDS-PAGE, and then electroblotted onto nitrocellulose (using a Biorad electroblotter). The blots were then either used for immunodetection ('Western blot') or for an assay of DNA binding ('South-Western blot'). Western blots were done as follows: proteins separated on PAGE were electroblotted onto nitrocellulose using a Biorad electroblotter. After washing with PBS (Phosphate buffered saline, obtained from Oxoid Ltd., England) and 1% NP40 blots were incubated with appropriate dilutions of rabbit antibodies directed against the C-terminal 12 amino acids of TcA. After additional washing the primary antibodies were visualized using GAR/AP (Goat anti rabbit alkaline phosphatase conjugate, Promega) and NBT-BCip (Nitro-blue-tetrazolium and 5-bromo−4-chloro-3 indolylphosphate, Promega). South-Western blots were done following Roth et al.[13] as follows: after PAGE proteins were blotted onto nitrocellulose using a Biorad electroblotter. Then proteins were denatured using 50mM Tris-HCl pH8.3, 50mM DTT, 1mM EDTA, M Guanidine-HCL, during 1h at room temperature. The blot was then washed in

50mM Tris-HCl pH7.5, 2mM EDTA, 2mM DTT, 0.5M NaCl, 0.1% NP40, 10% glycerol, during 1 h at room temperature, and in a second step in the same buffer 24h at 4°C. During this time the proteins have a chance to renature. Then the blot was preincubated with 0.02%BSA, 30mM HEPES/NaOH pH8.0, 30′ at room temperature, and subsequently incubated with 'binding buffer': 30mM HEPES/NaOH pH7.5, 50mM NaCl, 5mM $MgCl_2$, 2mM DTT to which radiolabeled DNA was added. After 20′ at room temperature the blot was washed several times with binding buffer, and autoradiography was done to visualize DNA binding proteins.

RESULTS

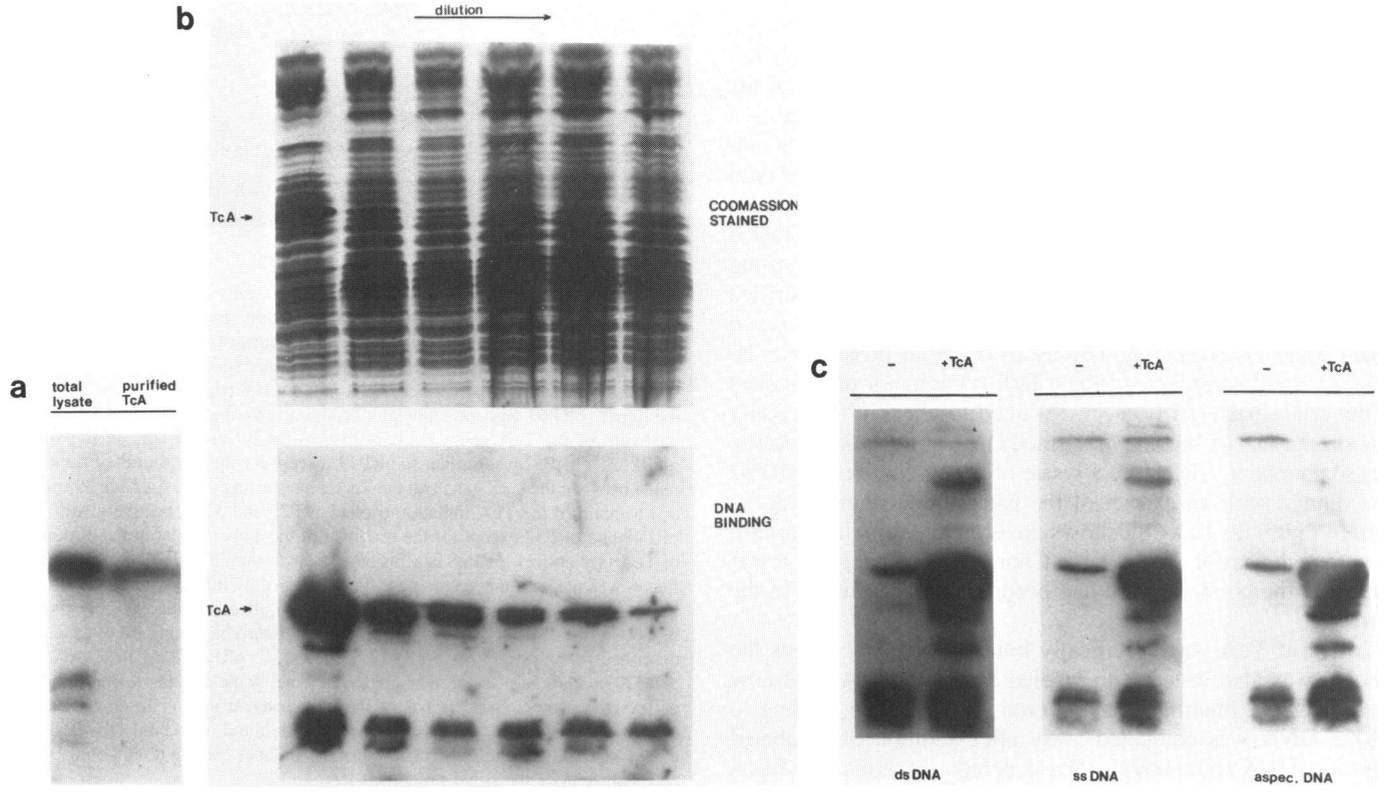
Overproduction of TcA in *E.coli*

Tc1 has one large ORF of 273 triplets. A second ORF nested in the larger gene in a different phase was described by Rosenzweig et al., (1983), but in a close homologue of Tc1 discovered in another nematode (*C.briggsae*) only the larger ORF is present (D.Baillie, A.Rose, pers.comm.) so it seems likely that only the large ORF is potentially encoding a protein. We called this putative protein TcA, and suggested that it might encode the transposase of Tc1[6].

The amount of TcA protein in nematodes is very small, probably too small to isolate the protein easily. This can be concluded from experiments that we did which will not be described here in detail: antibodies against peptides corresponding to the predicted TcA sequence were generated and found to be very specific in detecting TcA from recombinant source; these antibodies did not reproducibly recognize a specific protein in nematode extracts. These experiments are still in progress, but at this point we can already conclude that the amounts of TcA protein in the animal are very low. To obtain high inducible expression of TcA in *E.coli* the gene was cloned behind the tac-promoter (see Materials and Methods and legend to figure 1a). An SD sequence —needed for efficient translation initiation— was cloned upstream of the natural ATG triplet of TcA, so that the resulting protein is not a fusion product but has the correct N-terminus. As can be seen in figure 1b an *E.coli* strain containing the plasmid pRP67 expresses large amounts of TcA after induction with IPTG. This protein was not found without induction of the tac promoter, nor in a strain without the TcA plasmid.

Purification of TcA

After 4 h of induction TcA is the major protein found in cell lysates (see figure 1b). Like many proteins overproduced in *E.coli*



2 TcA is a DNA binding protein

a. A total lysate of *E.coli* strain JM101 containing the plasmid pRP67 after 4h of induction, and the partially purified TcA protein were run on a PAGE and blotted onto nitrocellulose. They were probed with pIM55 plasmid DNA radiolabeled by random priming. As can be seen there are some other proteins in the complete *E.coli* lysate that bind DNA; after purification TcA retains its DNA binding capacity.

b. A total lysate of JM101 containing pRP67 was diluted in a lysate of JM101 without this plasmid (in steps of 4). The leftmost lane contains an extract of JM101+pRP67, the following lanes are subsequent dilution steps and the rightmost lane is JM101 without the plasmid. As can be observed the TcA protein becomes less and less abundant in the dilution series; at 128× dilution the TcA protein is barely visible on the Coomassie stained gel, but still shows up prominently in the South-Western blot.

c. The three panels show a South Western blot of lysates of two strains: JM101, and JM101+pRP67. The probes were as indicated: ssDNA and dsDNA are pIM55 plasmid DNA labeled by random priming; in the former case they were and in the latter case they were not boiled briefly after the labelling. Aspec. DNA is salmon sperm DNA labeled by random priming.

Again the main DNA binding band in the lanes of JM101+pRP67 is TcA, and some other DNA binding bands can be seen which are present in both bacterial strains.

it is insoluble. The protein is not dissolved by addition of 0.1% SDS at room temperature or of 8M urea (not shown). However the protein is dissolved in 8M guanidine-HCl, a strong denaturing agent. We took advantage of this to obtain a one step purification of TcA. Cells were lysed; the debris was spun down and resuspended in a non-ionic detergent (0.1% NP40) in high salt (1M NaCl) which removes most cellular proteins. Then the remaining debris was dissolved in 8M guanidine-HCl, and the supernatant was cleared by centrifugation. The TcA in the supernatant is now almost pure. Direct removal of Guanidine-HCl by dialysis leads to precipitation of TcA. Therefore we dialyzed extensively against 8M urea to remove the guanidine, then stepwise against lower urea concentrations, until all urea was removed (allowing possible renaturation of the protein). This was done in the continuous presence of 1M NaCl. NaCl concentrations of 500mM or lower lead to precipitation of TcA, even in the presence of non-ionic detergents (results not shown). The purified TcA is shown in figure 1c: we estimate that it is approximately 80% pure. Similar purification protocols (involving denaturation and subsequent slow stepwise renaturation) have in many cases yielded active proteins; e.g. the phage Mu B transposase[14].

TcA is a DNA binding protein

The purified TcA was assayed for specific binding to Tc1 ends in gel retardation assays. No specific binding was observed (not shown); this is not surprising in view of the high salt concentration that is necessary to keep TcA in solution. Since the high salt concentration makes any attempt to detect DNA binding of the solubilized TcA likely to fail we used another technique.

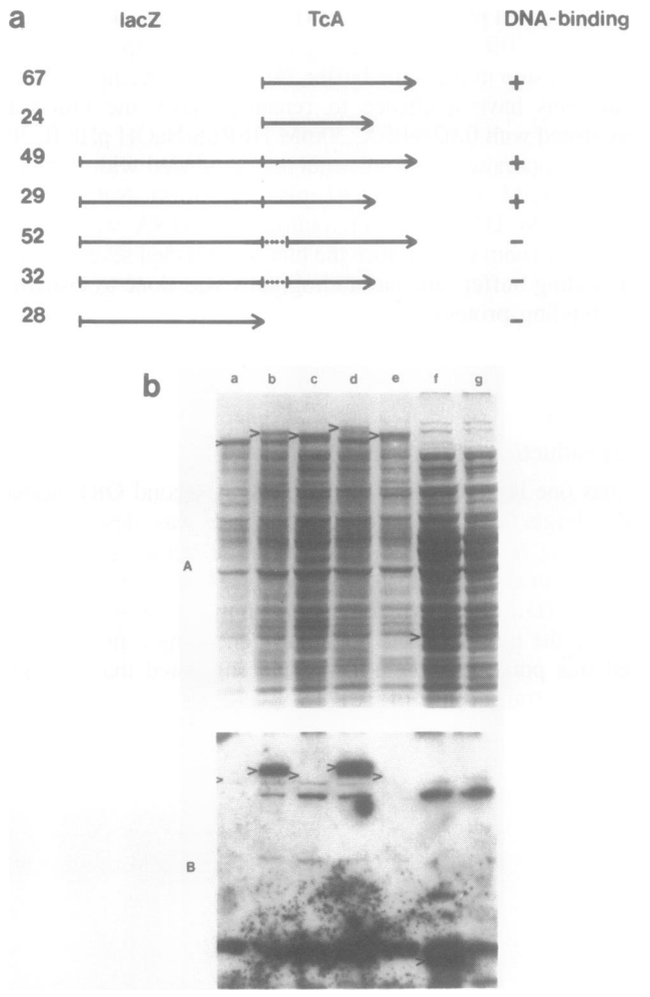
To assay DNA binding activity of TcA we performed a South-Western blot: the protein was run on a SDS-PAGE, blotted onto nitrocellulose, and incubated with radiolabeled DNA (of plasmid pIM55 containing a copy of Tc1). The plasmid DNA was labeled by random priming. As shown in figure 2a there is a very strong DNA binding activity found for TcA. This is true for the purified TcA, but also for the TcA in total *E.coli* lysate. In the total *E.coli* lysate some DNA binding activity of bacterial proteins can be seen, as could be expected. Since TcA is the major protein even in the crude bacterial lysate we wanted to check if TcA really was strongly DNA binding or seemed DNA binding only because it is so abundant. Therefore a lysate of *E.coli* containing pRP67 was diluted with an extract of the bacterium without TcA: as seen in figure 2b TcA still shows up as a DNA binding protein, even though —as observed in the Coomassie stained gel— it was diluted to the point where it had become a minor protein in the extract.

To test if TcA was specifically binding to the Tc1 ends the binding was also tested with labeled aspecific DNA, and now again efficient binding was observed (fig 2c). The binding to pIM55 DNA was competed away after addition of unlabeled aspecific DNA (not shown). We conclude that this TcA has a high DNA binding capacity, with no clear specific affinity for the sequences at the end of Tc1.

We tested if TcA had similar affinities for double stranded and for single stranded DNA. Binding of double stranded and single stranded probe were compared, and as shown in figure 2c both are bound by TcA.

A fusion protein β-gal/TcA binds DNA

The TcA gene was fused C-terminally to *lacZ* (see legends to fig. 3a) and tested in a South Western blot for DNA binding.



3. DNA binding by domains of TcA fused to β-gal
a. Fusion proteins of TcA and β-gal. The diagram shows the relevant portion of clones that contain either TcA, lacZ, and fusions of those two genes. Clone pRP67 and pRP24 are described in the text, the latter being identical to the former but for the presence of an amber triplet in TcA 108 triplets from the 3′ end of the gene[6]. pRP49 was the complete TcA gene (starting at the StuI site 20 bp upstream of the TcA gene) fused in fase to the 3′ end of lacZ in the vector pUR292[11]. pRP52 was similar to pRP49, except for the fact that the fusion was made between the lacZ gene and the TcA gene starting at the SalI site 39 triplets downstream of the TcA initiation triplet. pRP29 and 32 respectively were like pRP49 and pRP52 except for the presence of the amber triplet in the 3′ domain of TcA (see above). pRP28 is a fusion similar to that in pRP49, except that the fusion was made to the vector pUR291 instead of pUR292, which results in an out of frame fusion; therefore the protein seen after induction is only β-gal.
b. Coomassie stained gel (panel A) and South Western blot (panel B) of the clones described in 3b. a: pRP28; b:pRP29; c:pRP52, d: pRP49; e:pRP32; f:pRP67; g:JM101 without a plasmid. The arrows in panel A indicate the fusion proteins, or TcA; the arrows in panel B indicate the corresponding areas in the South Western blot. It is clear that the fusion proteins in lanes b and d do bind DNA, just as the TcA protein in lane f; the fusion proteins in lanes c and e do not bind DNA, and neither does the β-gal protein in lane a.

The result is shown in fig 3b lanes a and d: the fusion protein binds DNA. As a control an out of frame fusion of the two genes (which only produces β-gal) was tested: it shows no DNA binding. The binding of DNA to the fusion protein seems a little lower than to the integral TcA protein. The purified fusion protein showed no specific binding to Tc1 ends in a mobility-shift gel assay (results not shown; see also discussion).

Deletion derivatives of TcA tested for DNA binding

Many DNA binding proteins have a specific DNA binding domain that is functional as a more or less autonomous domain. To test whether the DNA binding properties of TcA could be assigned to a region of TcA we analyzed deletion derivatives of TcA. One of the naturally occurring derivatives is the TcA gene of the Tc1 isolate *st137* which was shown to be truncated by an amber codon in the C-terminal region of the gene[6]. An overproducer of this amber derivative did not yield high levels of the protein (results not shown); this may be due to the fact that the truncated protein is less efficiently expressed or proteolytically degraded in the bacterium. Deletion derivatives of the TcA gene fused to the C-terminal region of *lacZ* turned out to be expressed at high levels however. Fig. 3b shows a diagram of the various deletion derivatives of β-gal/TcA that were tested: a deletion of 39 amino acids at the N-terminus, a deletion of 108 amino acids at the C-terminus and the combination of both deletions. As shown in figure 3b panel A all fusion proteins are expressed at comparable levels. Western blots were made of all constructs using a serum raised against TcA; this confirmed the identity of the fusion proteins indicated by arrows in figure 3b panel A (not shown). The results of the binding test are shown in fig. 3b panel B: deletion of a large C-terminal segment of the protein does not strongly affect DNA binding, whereas a deletion at the N-terminal region does. Deletion of both segments also destroys the DNA binding capacity. These data demonstrate that the C-terminal region of TcA is not necessary for aspecific DNA binding, and suggest that the N-terminal region may be responsible for it.

DISCUSSION

Biochemical analysis of the mechanism of DNA transposition in animals has thus far proven to be difficult. A first prerequisite is a complete genetic description of the components essential for the transposition reaction; this has not yet been done in any animal system. The most detailed analysis has thus far been carried out for P-elements in *Drosophila* (see Rio et al.[15]), but also in that case purified transposase has not yet been shown to be active in vitro. The purpose of our work is to analyze the mechanism of Tc1 transposition in *Caenorhabditis elegans*.

Tc1 transposition in vitro

Thus far attempts to set up *in vitro* DNA recombination or transposition systems have depended on the availability of starting material that contained high levels of the recombinase or transposase enzymes (e.g. compare transposition of phage Mu[14] and inversion by Gin or Hin[16,17]. We have attempted to detect Tc1 rearrangements in nuclear extracts of nematodes (e.g. the 'high hopper' strain TR679[18]), but have not succeeded in detecting specific rearrangements above the background of random cutting and ligating in crude extracts (results not shown). Therefore we think the best approach to set up a system for analysis of Tc1 jumping *in vitro* would be to obtain the relevant factors from recombinant sources. At this stage it is not clear yet what all the relevant factors are. As described in the introduction it is very likely that the TcA gene product encoded by Tc1 is one of these factors. To test if TcA had properties corresponding with its assumed function as transposase of Tc1 we overproduced and partially purified TcA, and studied its properties.

The sequence of the TcA gene

The sequence of Tc1 was published in 1983[5] and thus far other copies of Tc1 have shown no apparently essential differences with the first sequenced element. We have sequenced the upstream regions of several different Tc1 elements of the Bristol N2 strain (results not shown), and the complete sequence of several Tc1 elements from the Bergerac strain have been determined (I.Mori, personal communication), and again they all seem largely identical. They do however all differ from the published sequence in one respect: an extra T base after base number 361 from the Rosenzweig sequence. This one basepair insert could be important: we would like to draw the attention to a potential intron sequence upstream of TcA (between bp 404 and 468). The sequence has all the features of a *Caenorhabditis elegans* intron: the consensus splice acceptor and splice donor sequences and stretches of A and T nucleotides. Taking into account the extra base mentioned above a splice would bring two ATG sequences upstream of TcA in phase, and these could be potential translation initiation sites. If the intron would indeed be spliced out the encoded TcA protein could be 335 instead of 273 amino acids long. Alternative splicing has been shown to occur in mRNAs for transposases in plants (e.g. Masson et al.[19]) and in Drosophila (in which case it also has a clear regulatory function[15,20]. Further research will be needed to determine whether the potential intron upstream of TcA is indeed in some cases spliced out in the nematode. In this study we have assumed that the 273 triplet ORF encodes TcA.

Properties of TcA synthesized in *E.coli*

The TcA made in *E.coli* is insoluble, like many recombinant proteins overproduced in *E.coli*. To purify it we had to denature it and let it slowly renature. The renatured protein is soluble in 1M NaCl, but quickly precipitates in lower salt concentrations. This is not prevented by addition of DNA or non-ionic detergents. This denaturation/renaturation procedure has been shown to lead to enzymatically active proteins in several cases, but in interpreting our results with TcA it should be kept in mind that there is a chance that the protein only shows partial activity because it is not necessarily correctly folded. A similar caveat should be made for the fact that we overproduced one version of TcA; there are a few minor differences between different copies of TcA[6,7] and it is not inconceivable that one version is more active in specific reactions than another.

We showed that TcA has a very high affinity for double stranded and single stranded DNA. This is in agreement with the presumed function of TcA as a transposase of Tc1. The DNA binding region of TcA is probably in the N-terminal part. This is a region which is especially rich in basic amino acids; the many arginine residues could play a role in binding to DNA. Apart from the abundance of arginines we observe no motifs that are frequently found in DNA binding proteins, such as Zinc-fingers or helix-turn-helix motifs.

We have not been able to demonstrate any sequence specificity in the DNA binding of TcA to Tc1 ends. This could be the result of imperfect refolding of the recombinant protein as discussed above; alternatively a second protein other than TcA could be needed to confer the necessary site-specificity upon the Tc1 transposition. This would not be without precedent: the phage Mu transposon has two transposases, A and B, and for the plant transposon En-1 two proteins Tnp-A and Tnp-B have been implied[21]. The presence of a Tc1 element in a strain is not

sufficient for Tc1 transposition; the additional factor(s) (referred to as *mut*[4]) could regulate TcA expression, but alternatively could encode a second protein that might play a role in Tc1 transposition and perhaps confer site-specificity on the system.

ACKNOWLEDGEMENTS

Janet Ampt, Henri van Luenen and Arjen Bakker contributed in initial stages of this work. Clones, strains, and many useful suggestions were contributed by Drs. I.Mori, B.Waterston, D.Moerman, P.Anderson, A.Rose and D.Baillie and S.Emmons. Dr J.Hodgkin is thanked for many fruitful discussions. One of us (RHAP) thanks Dr. J.Sulston for his hospitality during the months spent in his lab. The work was supported by a C&C Huygens fellowship from the Netherlands Organization for Scientific Research (NWO) to RHAP.

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