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Helix stability and the mechanism of cruciform extrusion in supercoiled DNA molecules

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ABSTRACT.

The kinetic properties of cruciform extrusion in supercoiled DNA molecules fall into two main classes. C-type cruciforms extrude in the absence of added salt, at relatively low temperatures, with large activation energies, while S-type cruciforms exhibit no extrusion in the absence of salt, and maximal rates at 50 mM NaCl, with activation energies about one quarter those of the C-type. These diverse properties are believed to reflect two distinct pathways for the extrusion process, and are determined by the nature of the sequences which form the context of the inverted repeat. C-type kinetics are conferred by A+T rich sequences, implying a role of helix stability in the selection. In this study we have shown that:

1 Helix-destabilising solvents (dimethyl formamide and formamide) facilitate extrusion by normally S-type molecules at low temperatures in the absence of salt.
2 C-type extrusion is strongly suppressed by low concentrations (2-4 μM) distamycin, at which concentrations S-type extrusion is enhanced.
3 Some extrusion occurs in a C-type construct in the presence of 50 mM NaCl. This is increased by addition of 3 μM distamycin, under which conditions extrusion becomes effectively S-type.

Thus S-type constructs can behave in a quasi-C-type manner in the presence of helix-destabilising solvents, and C-type extrusion is suppressed by binding a compound which stabilises A+T rich regions of DNA. Helix destabilisation leads to C-type behaviour, while helix stabilisation results in S-type properties. These studies demonstrate the influence of contextual helix stability on the selection of kinetic mechanism of cruciform extrusion.

INTRODUCTION.

DNA structure exhibits considerable potential for sequence-dependent local variability at a number of levels. Some of these, such as the formation of cruciforms [1-3] or left-handed Z-DNA [4], must be regarded as structural perturbations on a major scale. In order to possess a full understanding of such alternative conformations, and to assess any possible cellular role, it is necessary to describe the kinetic pathways by which they are interconverted.

The formation of a cruciform structure by an inverted repeat must of necessity involve a great deal of structural disruption, since it requires a complete reorganisation of the base-pairing. Initial kinetic studies [5] revealed that considerable kinetic barriers could exist, resulting in slow rates of extrusion even at elevated temperatures. In subsequent studies [6-9] no simple consensus on the general kinetics of cruciform extrusion emerged - in fact many of the results obtained in different systems appeared to be contradictory. However, in a systematic comparison of two plasmids containing different inverted repeats, we were able to show that cruciform extrusion can differ

widely in its kinetic character, resulting from differences in DNA base-sequence [10].

We have described two kinetic classes of cruciforms, with strongly contrasting properties:

C-type: C-type cruciforms are extruded in the absence of added salt, and the process is strongly suppressed by addition of salt. Extrusion is extremely temperature-dependent, corresponding to an Arrhenius activation energy in excess of 150 kcal mol^{-1}. Notwithstanding, extrusion of C-type cruciforms may occur at relatively low temperatures, half-times of a few minutes at 28°C being common.

S-type: S-type cruciforms are totally refractory to extrusion in the absence of salt, and exhibit maximal rates of extrusion at 50-60 mM monovalent cation (at which concentration C-type cruciform extrusion is 90% suppressed). Under these conditions extrusion proceeds at measurable rates at higher temperatures (typically above 35°C) with a temperature dependence corresponding to an Arrhenius activation energy of 30-50 kcal mol^{-1}.

The C-type behaviour is rather rare, the only known natural example being ColE1, while most other cruciforms studied in this laboratory and elsewhere are best described as S-type.

The differences between the two classes are so pronounced that they are thought to reflect two alternative kinetic pathways for extrusion [10-13], reviewed in Lilley *et al* [14]. We believe that extrusion of the C-type species proceeds *via* a large unpaired region, covering at least the entire inverted repeat, which then forms the fully extruded cruciform in a single event of hairpin formation. In contrast, the S-type pathway appears more complex, involving a more limited initial unpairing at the centre of the inverted repeat, followed by the formation of a partially extruded 'proto-cruciform', which then branch migrates to the complete structure. Systematic studies of the effects of base changes on S-type extrusion have indicated that the initial helix opening is restricted to the central region of the inverted repeat [13], and detailed analysis of the effects of cation size on S-type extrusion rates have supported the existence of a species akin to the proto-cruciform [12].

Recently we have demonstrated [11] that the critical factor in conferring C- or S-type kinetic character is the nature of the sequences flanking the inverted repeat. The ColE1 inverted repeat [2,3] exists in a context which is abnormally A+T rich. It emerged that these sequences could confer C-type kinetics on any inverted repeat placed at this location. Moreover the inverse was also true, for the ColE1 inverted repeat became S-type when introduced at the *Bam*HI site of pAT153, the site at which the bke cruciform of the archetypal S-type plasmid pIRbke8 normally resides. Interestingly, mixed-flank constructs - inverted repeats flanked by sequences of opposed kinetic influence - exhibited a character which was determined by the prevailing salt concentration. Thus the temperature dependence of these species was C-type in 0 mM and S-type in 50 mM NaCl added to the extrusion incubation. These results point to an intimate involvement of DNA sequence and cation concentration in the selection of cruciform extrusion mechanism.

All the sequences found so far which successfully confer C-type character are very A+T rich. Such sequences become denatured at relatively low temperatures [15-19], and suggest that their role may be associated with locally lowered helix stability. We have already proposed [11] that the contextual

influence may be related to telestability effects observed in thermal melting experiments [20]. We reasoned that it might be possible to examine this idea in greater detail by using agents which increase or decrease DNA helical stability, studying their effects upon the kinetic character of cruciform extrusion. By using helix-destabilising solvents (formamide and dimethyl formamide) and a compound which stabilises A+T rich DNA (distamycin) we have been able to demonstrate that destabilisation of the helix leads to C-type properties, while stabilisation leads to S-type character. These results underline the critical role of helix stability in the selection of the kinetic pathway for cruciform extrusion.

EXPERIMENTAL.

Plasmid constructions. Details of the construction of all of the plasmids employed in this study have been described previously: pColIR315 [21] and pIRCol/vec, pIRxke/col and pIRxke/vec [11]. The relevent sequences of all plasmids used in these studies have been confirmed by chemical

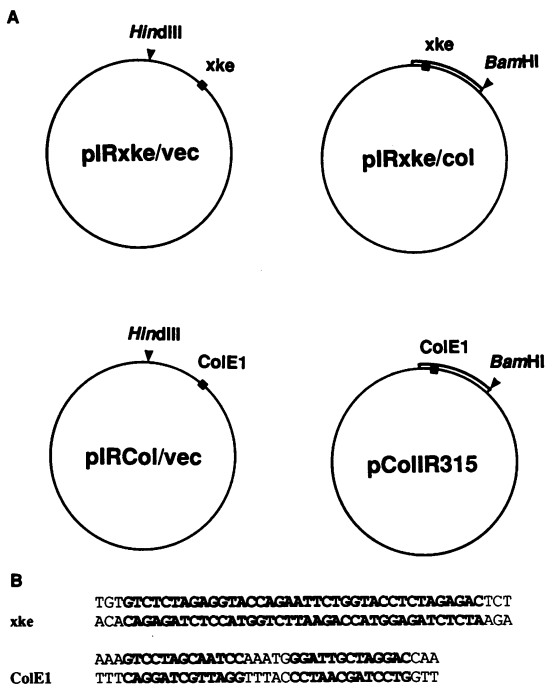

Figure 1. The plasmids employed in these studies. **(A)** Maps of the four plasmids, showing the location of the inverted repeats (filled boxes) in relation to the restriction sites used in the assay of relative extents of cruciform extrusion. The A+T rich ColE1 sequences are represented by the open regions. **(B)** The base sequences of the xke and ColE1 inverted repeats. Sequences related by two-fold symmetry are in bold type.

Table 1. Summary of the construction and kinetic character of the plasmids employed in these studies. For complete details of constructions, see [11].

Plasmid	Inverted repeat	Flanking sequences	Kinetic class
pIRxke/vec	xke	vector (pAT153 *Bam*HI site)	S
pIRxke/col	xke	ColE1	C
pIRCol/vec	ColE1	vector	S
pColIR315	ColE1	ColE1	C

sequencing [22]. The significant kinetic details of these plasmids are summarised in Figure 1 and Table 1.

Preparation of cruciform-free DNA. Cruciform-free plasmid DNA was prepared as previously described [10]. In brief, *Escherichia coli* cells harbouring the appropriate plasmids were cultured in minimal media to an A_{600} of 0.6 - 0.8, and subsequently chloramphenicol amplified for 16 hours. Cells were then lysed by the addition of lysozyme, SDS and EDTA and the DNA recovered was subjected to two rounds of isopycnic centrifugation on CsCl/ethidium bromide density gradients. Plasmid DNA recovered from the second gradient was extracted repeatedly with butan-1-ol at 0°C to remove the ethidium bromide, followed by extensive dialysis against pre-cooled TE buffer (10 mM Tris.HCl, pH 7.5, 0.1 mM EDTA) at 7°C. This DNA was then used directly for kinetic experiments, thus avoiding helix destabilising conditions, including exposure to elevated temperatures and organic solvents.

Chemicals. Formamide, dimethyl formamide and distamycin were obtained from Sigma.

Kinetics measurements. Kinetics experiments were carried out using 29 μg/ml cruciform-free DNA (typically 500 ng per sample). All incubations contained 10 mM Tris.HCl, pH 7.5, with the addition of NaCl, formamide, dimethyl formamide or distamycin as indicated in the results section. We have previously shown that over the temperature range employed, the temperature-dependent buffering by Tris.HCl does not have any measurable effect on the data obtained [11].

Incubations were performed in a Haake D8 thermostated bath in which the temperature was controlled to an accuracy of better than ± 0.05 C deg. Following a 5 minute period of incubation at a given temperature, an aliquot or sample was transferred immediately to ice, and allowed to cool. The relative degree of cruciform extrusion was then determined by S1 nuclease digestion, which cleaves specifically at the cruciform loops. Ten times reaction buffer was added to the DNA sample so that the final ionic conditions were 50 mM Na acetate, pH 4.6, 50 mM NaCl and 1 mM $ZnCl_2$. The sample was then incubated at 15°C for 20 minutes with 6 units of S1 nuclease (BRL). DNA was then ethanol precipitated and cut to completion with a restriction enzyme (*Bam*HI for pIRxke/col and pColIR315, and *Hin*dIII for pIRxke/vec and pIRCol/vec). In samples in which the

DNA had been incubated with distamycin, five times the normal quantity of restriction enzyme was added, in order to overcome the strong inhibition of restriction digestion caused by the antibiotic. The DNA fragments produced were analysed by electrophoresis on a 1% agarose gel in 90 mM Tris.borate, pH 8.3, 10 mM EDTA, at room temperature for 14 hours, and subsequent staining by 1μg/ml ethidium bromide. Following destaining in distilled water, gels were photographed under UV illumination using Kodak Tri-X Pan film.

Data analysis. The degree of cruciform extrusion was quantified by scanning laser densitometry of the photographic negatives of gels, using an LKB Ultroscan 2202 densitometer. The latter was interfaced to an Apple IIa computer for curve fitting and integration. We define the relative extent of cruciform extrusion as the intensity of the band due to S1 nuclease digestion at the inverted repeat divided by the total intensity. The units of extrusion are relative values only, scaled on a 1-4 range.

RESULTS.

The effects of helix-destabilising agents on S-type cruciform extrusion kinetics.

pIRxke/vec is a plasmid containing a typical salt-dependent (S-type) cruciform. We have previously characterised the kinetic properties of the extrusion of the xke inverted repeat in supercoiled pIRxke/vec [11], and demonstrated optimal rates of cruciform formation at 50 - 60 mM NaCl. In the absence of added salt there is no measurable cruciform extrusion even after prolonged incubation at 45°C. Consideration of the proposed mechanisms of cruciform extrusion [10-13] induced the suspicion that DNA helix stability might be an important factor in pathway selection, and as a consequence we decided to examine the effect of helix destabilising solvents on the kinetic character of this S-type cruciform. Solvents such as formamide significantly reduce the melting temperature of DNA [23].

We chose to study the influence of the duplex destabilising agents formamide and dimethyl formamide (DMF) on the extrusion kinetics of pIRxke/vec. It is important to note that there was no salt added to these incubations, which would normally be essential in order to achieve extrusion of this cruciform [12]. Aliquots of cruciform-free DNA [10] were incubated at 22°C for 5 minutes in the presence of 10 mM Tris.HCl pH 7.5 with increasing amounts of either reagent, to a maximum concentration of 75% by volume. Following the incubation, the samples were returned to ice, allowed to cool. The relative extent of cruciform extrusion was assayed by S1 nuclease digestion at 15°C, subsequent complete cleavage by the restriction enzyme *Hin*dIII, and analysis of the cleavage products by agarose gel electrophoresis. Photography of the ethidium bromide stained gels and scanning laser densitometry of the negatives allowed the relative degree of cruciform extrusion to be quantified. The data are presented in Figure 2. While no extrusion occured in the course of the incubation in the absence of formamide or DMF, with increasing quantities of the reagents extrusion becomes facilitated, although higher concentrations caused the extrusion to disappear again.

It is clear that both helix destabilising reagents are able to induce a measurable degree of cruciform

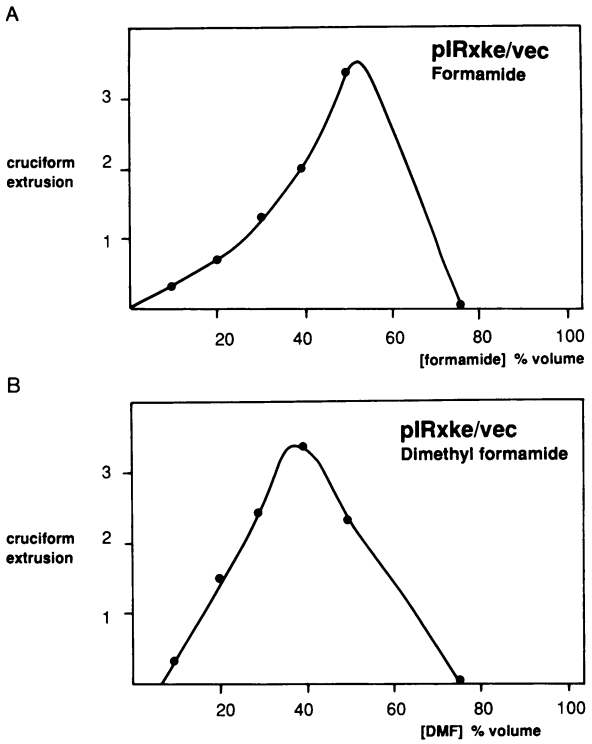

Figure 2. Extrusion of an S-type cruciform in the presence of helix destabilising solvents. Relative extrusion of pIRxke/vec as a function of formamide and dimethyl formamide concentrations. **(A)** Relative extent of cruciform extrusion by pIRxke/vec occuring during a 5 m incubation at 22°C as a function of formamide concentration. DNA samples were incubated at 22°C for 5 minutes in the presence of 10 mM Tris.HCl, pH 7.5, and the indicated concentration of formamide, then cleaved with S1 nuclease at low temperature and finally cut to completion with *Hin*dIII. The DNA was electrophoresed in 1% agarose, and the relative extent of extrusion estimated by laser densitometry of gel photographs. **(B)** Relative extent of cruciform extrusion by pIRxke/vec occuring during a 5 m incubation at 22°C as a function of dimethyl formamide concentration. The experiment was carried out exactly analogously to that using formamide.

extrusion in the complete absence of salt, from an inverted repeat whose extrusion would normally require the presence of added cations. They are able to impose a kinetic character - salt-independent extrusion - on an S-type inverted repeat, that is normally characteristic of C-type cruciform extrusion. Both reagents facilitate cruciform extrusion in the absence of salt, and both exhibit maxima. The optimal concentrations of DMF and formamide were slightly different - 40% and 50% respectively, and the absolute level of extrusion induced by DMF was also slightly higher than that seen with formamide. For these reasons, DMF was used for all further experiments.

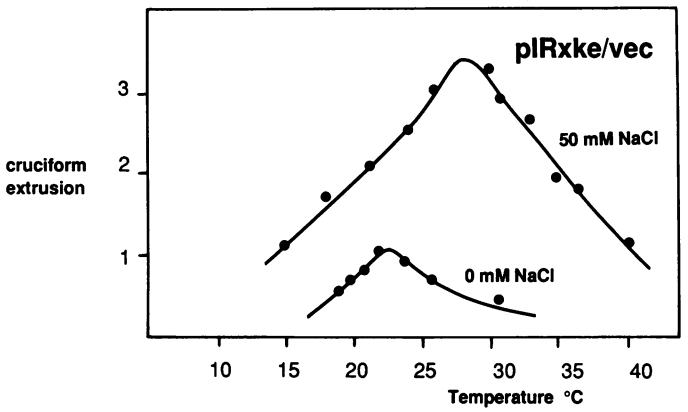

Figure 3. Temperature dependence of cruciform extrusion of a S-type plasmid in the presence of 40% dimethyl formamide. Cruciform-free pIRxke/vec was incubated in the presence of 10 mM Tris.HCl, pH 7.5, 40 % DMF with either 0 or 50 mM NaCl. DNA was exposed to the indicated temperature for 5 minutes, prior to assaying for cruciform extrusion.

Temperature dependence of an S-type cruciform extrusion in the presence of 40% DMF - conversion of S- to C-type character.

The ability of DMF to facilitate pIRxke/vec extrusion in the absence of salt suggests that the helix-destabilising agent may cause changes in the local DNA structure which mimic the effects of C-type inducing sequences. It was therefore of interest to see whether the pseudo-C-type kinetic character imposed by DMF would extend to the temperature dependence of cruciform extrusion from the 'xke' inverted repeat. Under conventional S-type assay conditions, xke cruciform extrusion is moderately temperature dependent, and occurs over the 30-50°C temperature range. By contrast, C-type cruciforms exhibit a much greater temperature dependence, and the extrusion proceeds at a lower temperature, typically 27-30°C.

To determine the influence of DMF on the temperature dependence of the extrusion, aliquots of cruciform-free pIRxke/vec were incubated with 10 mM Tris.HCl pH 7.5, 40% DMF for 5 minutes over a wide range of temperatures. The temperature profile obtained is shown in Figure 3, together with the data from an exactly equivalent experiment carried out in the presence of an additional 50 mM NaCl. The results demonstrate that in the presence of DMF, cruciform extrusion is markedly temperature-dependent, and that significant rates of extrusion occur at relatively low temperatures. These properties are characteristic of normal C-type kinetics, and suggest that agents which destabilise random DNA sequences of average base composition may mimic the effects of A + T rich DNA on the extrusion kinetics. The results differ from those found for a typical C-type cruciform, such as that of pIRxke/col, under normal conditions in that beyond a critical temperature, 22°C in the absence of salt, the extent of cruciform extrusion fell once again.

The addition of 50 mM NaCl increased the amount of cruciform extrusion occuring during the fixed period of the incubation, and shifted the temperature profile towards higher temperatures. The greater degree of extrusion in the presence of salt could be a result of cations continuing to act in their normal role in stabilising the transition state [12]. More probably, the effect could arise from salt acting antagonistically to DMF, stabilising the duplex structure at higher temperatures. This last possibility could explain why the extrusion exhibits a distinct maximum in the presence of both 0 and 50 mM NaCl - it is most likely due to a thermodynamic rather than a kinetic effect. It is possible that in the presence of DMF, the duplex begins to unwind and melt at elevated temperature, to such an extent that the level of superhelix density falls to a level below that which may support cruciform formation.

To test this hypothesis we performed the following control experiment. Two samples of cruciform-free pIRxke/vec DNA were incubated in 10 mM Tris.HCl pH 7.5, 40 % DMF at 22°C for 15 minutes, after which one sample was returned to ice while the second was incubated for a further 15 minutes at 45°C. Each was then assayed for cruciform formation as normal. If the low level of extrusion at 45°C was due to a slow rate of extrusion at this temperature, then both samples should show similar levels of extrusion, as they were both incubated at the optimal temperature for 15 minutes. By contrast, if the effect is thermodynamic, in that at elevated temperature the cruciform is unstable, then the sample heated to 45°C should show a smaller amount of extrusion than the sample that was returned to ice. In fact we observed that the level of cruciform extrusion in the 45°C treated sample was only about 15% that of the control sample, showing that the cruciform must indeed be thermodynamically unstable at elevated temperatures in the presence of 40% DMF. Thus only the temperature profile up to the extrusion maximum can be considered representative of a kinetic effect, above that temperature, thermodynamic effects become appreciable.

The effect of helix-stabilisation on cruciform extrusion kinetics.

If agents which destabilise the double helix may induce pseudo-C-type kinetics in a cruciform located in random sequence DNA, then conversely, factors which stabilise A + T rich DNA might hinder C-type extrusion. The antibiotic distamycin A [24] binds A + T regions specifically with high affinity, stabilising and rigidifying the duplex structure [25]. NMR studies [26] have shown that it locates in the minor groove, analogously to netropsin binding, which has been demonstrated by crystallographic studies [27]. We therefore decided to examine the effect of binding this drug on C-type cruciform extrusion kinetics.

A potential complication with this molecule is the possibility that it will bind the A+T rich central loop regions of the inverted repeats under study, hindering cruciform formation. To determine the extent to which this inhibits cruciform extrusion, experiments were carried out in parallel on the plasmids pIRxke/vec (S-type) and pIRxke/col (C-type). These molecules represent different kinetic classes and yet possess the same inverted repeat sequence. Hence any difference in the extrusion of the two, as a function of distamycin concentration, must be due to the effects of distamycin binding to the flanking sequences, rather than to the inverted repeat.

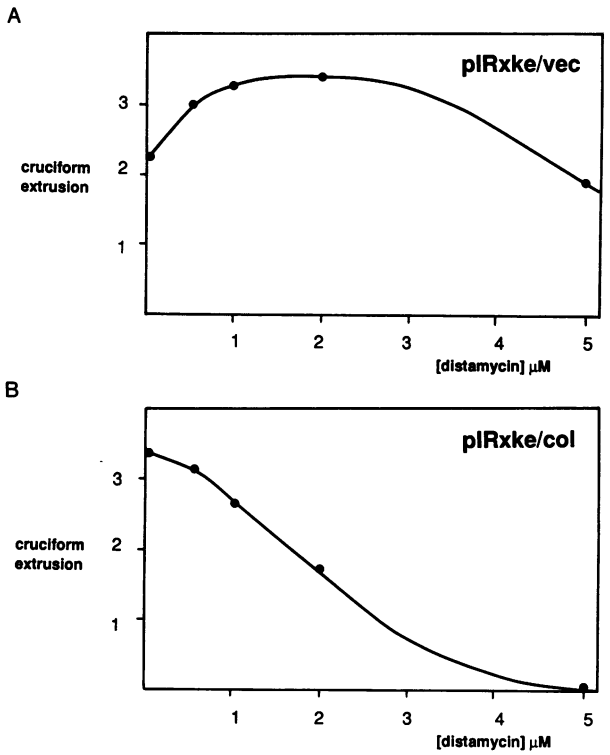

Figure 4. Effect of distamycin on the extrusion of S- and C-type plasmids containing the xke cruciform. **(A)** Extrusion of pIRxke/vec as a function of distamycin concentration. DNA samples were incubated in the presence of 10 mM Tris.HCl, pH 7.5, 50 mM NaCl and the indicated distamycin concentration for 5 minutes at 45°C. They were subsequently assayed for cruciform extrusion as normal. **(B)** Extrusion of pIRxke/col as a function of distamycin concentration. The experiment was carried out as described in (A), except that no NaCl was added.

Initial experiments indicated that at distamycin concentrations of >10 μM, there was complete inhibition of cruciform extrusion from both plasmids. At lower concentrations, however, the extrusion of the two plasmids as a function of distamycin concentration differed greatly.

The results are presented graphically in Figure 4. They show that the extrusion of pIRxke/col, a C-type inverted repeat, is partially suppressed even by the addition of 0.5 μM distamycin, and that by 5 μM, there is no extrusion whatsoever. In contrast, binding of low concentrations of distamycin to pIRxke/vec, an S-type construct, actually stimulates extrusion, with an optimum at 3 - 4 μM. Thus distamycin concentrations in the 0 - 5 μM range produce completely contrasting behaviour in the kinetic character of C- and S-type cruciform extrusion. While the C-type extrusion is strongly suppressed, S-type extrusion is slightly enhanced. The suppression of the C-type extrusion by distamycin suggests that the antibiotic binds to the A + T rich flanking sequences, and that the resulting helical stabilisation inhibits the normal kinetic influence being felt by the neighbouring inverted repeat.

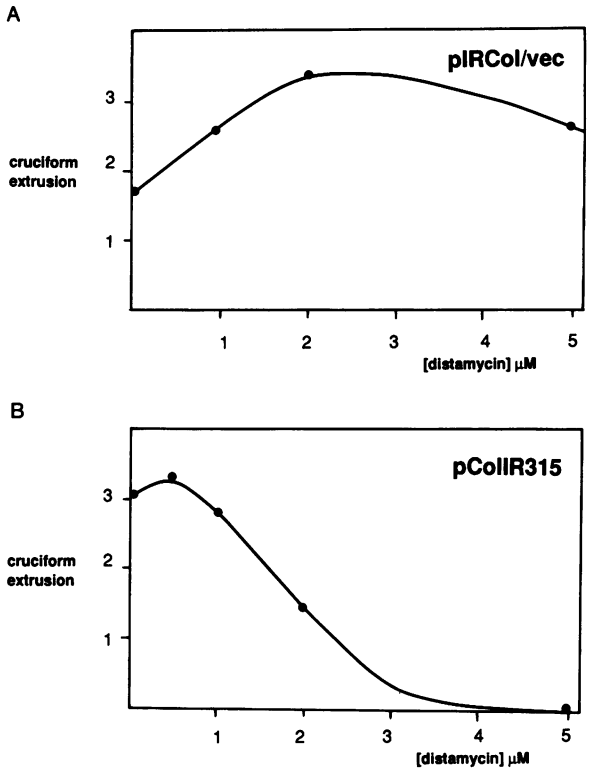

Figure 5. Effect of distamycin on the extrusion of S- and C-type plasmids containing the ColE1 cruciform. **(A)** Extrusion of pIRCol/vec as a function of distamycin concentration. DNA samples were incubated in the presence of 10 mM Tris.HCl, pH 7.5, 50 mM NaCl and distamycin at the concentrations indicated. All samples were incubated at 45°C for 5 minutes prior to assay for cruciform extrusion. **(B)** Extrusion of pColIR315 as a function of distamycin concentration. The experiment was carried out as described in (A), except that no NaCl was added to the incubation.

Extrusion of pColIR315 and pIRcol/vec as a function of distamycin concentration.

To ensure that the data obtained with the xke inverted repeat in C- and S-type flanking sequences were not due to some peculiarity of that inverted repeat sequence, the experiments were repeated using pColIR315 and pIRcol/vec, in which the ColE1 inverted repeat is present in C- and S-type environments respectively.

The results with these plasmids were almost identical to those obtained with the xke constructs. Once again there was complete suppression of extrusion in both plasmids at distamycin concentrations >10 μM. The extrusion profiles over 0 - 5 μM distamycin are shown in Figure 5. The C-type kinetics were completely suppressed by 4 - 5 μM, whilst the S-type cruciform showed an increase in extrusion up to ~3 μM distamycin, beyond which extrusion diminished at 8 - 10 μM. It is possible that the differential effects of distamycin on C- and S-type cruciform extrusion might

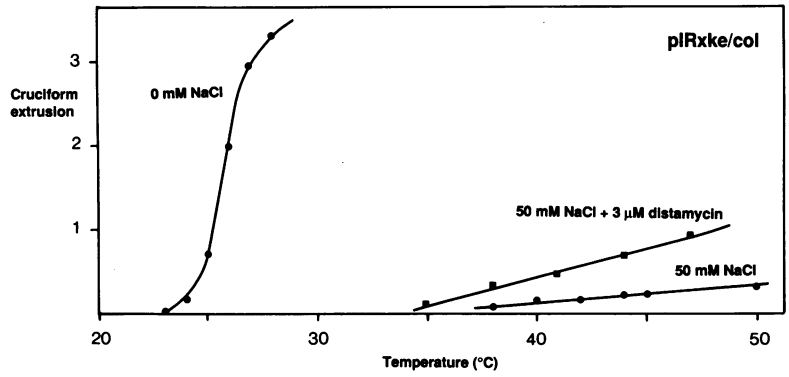

Figure 6. A C-type cruciform showing S-type kinetic properties - the combined effects of salt and distamycin. Cruciform-free pIRxke/col was incubated as function of temperature in the presence of 10 mM Tris.HCl, pH 7.5, alone or plus 50 mM NaCl, or plus both 50 mM NaCl and 3 μM distamycin. Samples were incubated at the indicated temperatures for 5 minutes, or 10 minutes for the incubation in Tris.HCl, pH 7.5, 50 mM NaCl. The latter was necessitated by the very low degree of extrusion occuring under these conditions, and we have halved the measured extrusion so that the data are comparable with the other data presented in this Figure.

be simply a consequence of the 0 and 50 mM NaCl concentrations respectively present in the incubations, which may have affected the affinity of distamycin binding. To examine this possibility, the following control experiment was performed. pColIR315 and pIRcol/vec were each incubated in 10 mM Tris.HCl pH 7.5, 20mM NaCl and 3 μM distamycin. At this salt concentration both plasmids should exhibit some extrusion, although it is optimal for neither cruciform. Under these conditions we observed that the C-type extrusion was suppressed whilst the S-type was not, showing that the differences between the two were *not* a consequence of the salt concentration. Since both the salt concentration and the sequence of the inverted repeat remained constant it follows that the differences must have arisen from distamycin exerting differential effects on the two sets of flanking sequences.

Extrusion of pIRxke/col as a function of temperature in the presence of both NaCl and distamycin - conversion of C- to S-type character.

We have demonstrated above that destabilising DNA structure with solvents such as DMF can confer quasi-C-type character on otherwise S-type cruciform-containing molecules. This naturally poses the question, could the inverse be possible? In mixed-flank constructs, that is to say inverted repeats flanked by one S-type and one C-type sequence, the kinetic character is C-type in 0 mM NaCl and S-type in 50 mM NaCl. In pure C-type constructs cruciform extrusion is strongly suppressed by the addition of NaCl, this being virtually total by 100 mM NaCl [10]. It had remained a source of some puzzlement to us [11] why these molecules did not default to S-type behaviour in 50 mM NaCl, similar to mixed-flank constructs. In point of fact, despite the

suppression of cruciform extrusion of the pure C-type species, such as pColIR315 and pIRxke/col, there remains a low level of extrusion in 50 mM NaCl - at about 10% of that in the absence of salt. We have examined the kinetic character of this residual level of extrusion in further detail.

The temperature dependence of cruciform extrusion by pIRxke/col in the presence of 0 and 50 mM NaCl is presented in Figure 6. In the absence of salt, the profile is typical of a C-type molecule, extrusion occuring at relatively low temperatures but with a high temperature dependence. By employing longer incubation times and higher temperatures, we have been able to study the extrusion in the presence of 50 mM NaCl. We found that extrusion occured to a measurable extent at relatively high temperatures, ie 35-50°C, and with a low temperature dependence. This is effectively S-type character, although the extrusion process is clearly rather reluctant under these conditions. At this point we considered the possibility that further stabilisation of the flanking regions might conceivably increase the rate of extrusion, and we therefore tried the addition of 2-4 μM distamycin to the incubation. It can be seen in Figure 6 that the addition of 3 μM distamycin does indeed facilitate the extrusion of pIRxke/col in 50 mM NaCl, by a factor of about 3 for any given temperature. 2 and 4 μM distamycin were somewhat less effective. The fact that 3 μM was the most effective concentration is consistent with the data of Figure 4, where 3 μM distamycin induced near-maximal rates of S-type extrusion in pIRxke/vec.

It appears that the stabilisation of the ColE1 flanking sequences resulting from the binding of distamycin leads to a marked enhancement of S-type extrusion in this system.

DISCUSSION.

We have presented two significant observations concerning the kinetic properties of cruciform extrusion.

1. Addition of helix-destabilising solvents into incubations of S-type cruciform constructs may lead to extrusion occuring in the absence of salt, and at relatively low temperatures.
2. C-type extrusion in suppressed by binding low concentrations of distamycin. Stabilisation of DNA structure by salt and distamycin may result in extrusion, but at relatively elevated temperatures, and with a much reduced temperature dependence.

These results point to an intimate involvement of DNA helix stability in the selection of cruciform extrusion mechanism. We have proposed previously [10-13] that extrusion of cruciform structures may proceed *via* two possible mechanisms. In the S-type mechanism, the process initiates at the centre of the inverted repeat to form a transition state which is probably well represented by a partially extruded cruciform [12,13], and which to a first approximation is independent of the sequences flanking the inverted repeat. The C-type mechanism is characterised by a transition state which resembles a large unpaired 'bubble' encompassing the entire inverted repeat. There is a critical involvement of the flanking sequences in this mechanism, for which the presence of at least one A+T rich sequence is required. A+T rich sequences have a lower helical stability than those of higher G+C content [15-19], suggesting that lowered DNA stability neighbouring the inverted

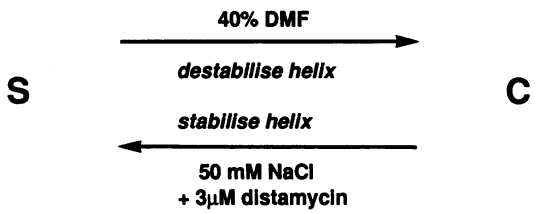

Figure 7. The interconversion of kinetic character of cruciform extrusion by perturbation of helix stability. The diagram summarises our conclusions from the data obtained from the investigation of the action of helix-stabilising and destabilising agents on cruciform extrusion kinetics. Agents which destabilise a normal duplex structure (e.g. 40% dimethyl formamide) may mimic the effects of C-type inducing sequences, and impose quasi-C-type kinetics on a normally S-type inverted repeat. Conversely, agents which bind and stabilise A+T rich duplex structure (50 mM NaCl + 3 μM distamycin), such as that proposed for C-type inducing elements, will inhibit C-type extrusion and favour an S-type mechanistic pathway for extrusion.

repeat may influence the kinetic character of the transition, such that the C-type pathway becomes operative.

On the basis of the mechanistic models proposed for C- and S-type cruciform extrusion, and the involvement of the flanking sequences in the former case, some simple predictions were possible. General loosening of the helix should induce S-type molecules to become more C-like, and stabilisation of the A+T rich flanking sequences of C-type molecules should make them become more S-like. In this study we have tested these two predictions, and shown them to be correct. In general terms, lowering helix stability artificially by means of organic solvents leads to quasi-C-type kinetic character, while increasing the stability with distamycin suppresses extrusion by the C-type mechanism. A combination of the optimal concentrations of salt and distamycin leads to quasi-S-type character in a normally C-type species. By these twin perturbations of helix stability we may transform at will between C-type and S-type kinetic properties, summarised in Figure 7.

We note that the maximal rate of extrusion for the S-type molecules in the absence of salt is brought about by 50% formamide at 22°C. It may be instructive to think about this in terms of the reduction in melting temperature (T_m) of the DNA brought about by the solvent. An empirical formula relates the T_m of a given sequence of DNA to its base composition and the sodium ion concentration [28]

$$T_m = 81.5°C + 0.41\ (\%G+C) + 16.6 \log[Na^+].$$

Thus the flanking sequences of pColIR315 and pIRxke/col will have a T_m about 20 degrees lower than those of pIRxke/vec in 10 mM Tris.HCl. The reduction of T_m by formamide has also been the subject of a number of empirical studies [23,29], leading to the relationship

$$T_m = T_m° - 0.62\ (\%\text{formamide})$$

where $T_m°$ is the T_m in the absence of the solvent. Thus 50% formamide will reduce the T_m of the S-type flanking sequences by about 31 degrees, ie they will become less stable than the C-type sequences without added formamide. This is consistent with the observed quasi-C-type extrusion at low temperature in the presence of 50% formamide. A simple application of the formulae to the

flanking sequences of pColIR315 and pIRxke/vec suggests that their kinetic properties may become most closely similar when 35% formamide is added to the latter.

One aspect of the effects of distamycin binding merits further discussion. It was seen that extrusion by the C-type species pIRxke/col in 50 mM NaCl was actually *improved* by addition of 3 μM distamycin. Furthermore, 2-3 μM distamycin facilitated the extrusion of S-type cruciforms, such as pIRxke/vec, in the same salt concentration, while strongly suppressing that of pIRxke/col in 0 mM NaCl. This suggests that a role does exist for the flanking sequences in S-type extrusion, though it may be a negative one. From these data it appears that further stabilisation of flanking sequences by low concentrations of distamycin leads to more efficient extrusion by the S-type mechanism. How can this be, if the S-type mechanism is solely dependent on local effects at the centre of the inverted repeat? Perhaps for maximal efficiency of S-type extrusion, it is necessary to concentrate torsional stress at the region which is to be opened initially. This will be helped by events leading to stabilisation of sequences elsewhere in the supercoiled molecule, and remote sequences with a predisposition to unwinding will act antagonistically. Under torsional stress, the sequence having the lowest torsional rigidity will deform first, and stiffening such sequences will help to concentrate the stress elsewhere. Distamycin is probably particularly effective, since it stabilises A+T rich regions due to its sequence preferences [24-26], and therefore selectively rigidifies the most probable torsional competitors for the inverted repeat.

In a sense we arrive at a new way of regarding the distinction between S- and C-type cruciform extrusion mechanisms, based on the deformability of the DNA in and around the inverted repeat. To achieve extrusion *via* the S-type mechanism it is necessary that the local sequence is sufficiently rigid that the inverted repeat is subjected to maximal torque. However, if the context of the inverted repeat is itself deformable, this raises the probability of formation of a much larger region of base-opening, and hence extrusion *via* the C-type pathway. This is consistent with the chemical reactivity of the flanking sequences in supercoiled C-type molecules [JC Furlong, KM Sullivan, AIH Murchie & DMJ Lilley in preparation].

These studies underline the necessity of taking a broader view of local sequence and its context when considering the dynamics of structural interconversion in supercoiled DNA molecules, and provide a potential source of *cis*-acting effects in DNA.

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