Quantitative comparison of DNA looping *in vitro* and *in vivo*: chromatin increases effective DNA flexibility at short distances

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The probability that two sites on a linear DNA molecule will contact each other by looping depends on DNA flexibility. Although the flexibility of naked DNA *in vitro* is well characterized, looping in chromatin is poorly understood. By extending existing theory, we present a single equation that describes DNA looping over all distances. We also show that DNA looping *in vitro* can be measured accurately by FLP recombination between sites from 74 bp to 15 kb apart. In agreement with previous work, a persistence length of 50 nm was determined. FLP recombination of the same substrates in mammalian cells showed that chromatin increases the flexibility of DNA at short distances, giving an apparent persistence length of 27 nm.

Keywords: chromatin/DNA looping/FLP recombination/
persistence length

Introduction

DNA looping plays a crucial role in many cellular processes, including replication, recombination and transcription (Bellomy and Record, 1990; Schleif, 1992; Dillon *et al.*, 1997). Looping efficiency depends on the flexibility of DNA or chromatin and the distance between the sites. Active mechanisms may also be involved. The looping behaviour of naked linear DNA is best described by a polymer model. The inherent stiffness of a polymer is expressed as its persistence length (P , in nm). The flexibility of naked DNA in aqueous solution has been well studied, establishing a persistence length of 50 nm (Hagerman, 1988). For a polymer of given stiffness, the probability that two sites on the same molecule will meet by looping can be expressed as the local molar concentration of one site with respect to the other (j_M , in M) (Kratky and Porod, 1949; Jacobson and Stockmayer, 1950; Hagerman, 1988; Rippe *et al.*, 1995). To calculate

j_M as a function of distance for linear DNA, two equations have been described, one applicable to short and the other to long distances. The Shimada–Yamakawa equation (Shimada and Yamakawa, 1984) is valid only for DNA fragments shorter than 2000 bp. Above this range, the freely jointed chain model is used (Flory, 1969; Rippe *et al.*, 1995). In Figure 1A, both curves are plotted, showing that neither model enables the calculation of j_M across the full range of DNA lengths. It is necessary to use an interpolation formula for the transition between the two curves (Shimada and Yamakawa, 1984).

Computer simulation of DNA based on polymer chain statistics offers an alternative approach for the analysis of looping probability (Hagerman, 1985; Hagerman and Ramadevi, 1990; Klenin *et al.*, 1998; Merlitz *et al.*, 1998). In principle, this approach could be used to determine j_M for the full range of distances. However, very large ensembles of chains are required to obtain reasonable chain statistics (Hagerman, 1988). The accumulation of statistical errors has limited computer simulations to chains of <470 bp (Klenin *et al.*, 1998). Each of the theoretical approaches currently available is limited to a given range of DNA lengths. However, DNA looping *in vivo* occurs from short distances to beyond the kilobase range. Clearly, a single coherent equation for j_M is required.

Experimental studies of DNA flexibility *in vitro* have made use of various methods, including ligase-catalysed ring closure (Shore *et al.*, 1981; Shore and Baldwin, 1983a,b), electro-optic methods (Porschke, 1991), electron microscopy (Bednar *et al.*, 1995) and scanning force microscopy (Rivetti *et al.*, 1996, 1998; Rippe *et al.*, 1997; reviewed in Bustamante *et al.*, 1994). The studies to date have been performed largely with DNA molecules of <1 kb. For this size range, the results are well described by the theoretical models. All consistently yield a persistence length of between 45 and 53 nm (reviewed in Hagerman, 1988) and an optimum distance for looping of ~500 bp.

In eukaryotic chromatin, factors such as supercoiling, wrapping of DNA around nucleosomes and higher order packaging may alter the effective flexibility of DNA. Estimations of DNA looping *in vivo* have been deduced from transcriptional assays (Bellomy *et al.*, 1988; Bellomy and Record, 1990; Law *et al.*, 1993; Dillon *et al.*, 1997; Fraser and Grosveld, 1998; Gribnau *et al.*, 1998) and chromosomal tagging (van den Engh *et al.*, 1992; Trask *et al.*, 1993; Marshall *et al.*, 1997). Transcriptional assays do not measure DNA looping directly, but are also partially dependent on transcription efficiency. For the β -globin locus control region (LCR), this drawback has been overcome by comparing functionally equivalent β genes at two different positions (Dillon *et al.*, 1997). However, this system is not suitable for the quantitative analysis of looping at both short and long ranges, firstly because the LCR itself spans 15 kb, and secondly because the

Fig. 1. Predictions of DNA looping. **(A)** Three models for calculating j_M as a function of the length of DNA between two sites interacting *in cis*. j_M is the local molar concentration of one site with respect to the other. Dotted line: the Shimada–Yamakawa model (1984), plotted using Equation 4 of Rippe *et al.* (1995). Dashed line: the freely jointed chain model plotted using Equation 3 of Rippe *et al.* (1995). Solid line: Equation 2 of the present study, valid for all DNA lengths. The persistence length, P , was taken as 50 nm in all three models. **(B)** Parameters for modelling a bound protein of diameter, r , were introduced into Equation 2 and fitted to data derived from Brownian dynamics simulations. Solid line: j_M calculated using Equation 2 (Materials and methods) for which $r = 0$. Open circles: j_M values derived from Brownian dynamics simulations with $r = 10$ nm (Rippe *et al.*, 1995; Merlitz *et al.*, 1998). Dotted line: j_M calculated using Equation 3 with $r = 10$ nm.

interactions of interest take place over distances of several kilobases. Similarly, chromosomal tagging techniques are limited by low resolution: ~100 kb for interphase chromosomes (Hahnfeldt *et al.*, 1993; Ostashevsky and Lange, 1994).

A systematic quantitative comparison of DNA looping *in vitro* and *in vivo* has not been undertaken previously, due to the lack of a single method that can be used in both systems, which gives a direct readout of the looping event and that is applicable to all DNA distances of interest. Here we report that these criteria are fulfilled by site-specific recombination catalysed by FLP recombinase. In FLP-mediated excision, two target sites (FRTs) are bound by FLP, synapsis takes place by random collision (Beatty *et al.*, 1986) and recombination results in the excision of the intervening DNA (reviewed in Sadowski, 1995). FLP recombination takes place efficiently *in vitro* and in a wide range of organisms *in vivo* (reviewed in Kilby *et al.*, 1993). Using a set of excision substrates with varying distances between their FRTs ranging from 74 bp to 15 kb, we show that FLP-mediated excision gives a quantitative measure of DNA looping on linear DNA at all distances both *in vitro* and *in vivo*. Whereas our *in vitro* results agree well with previous studies of DNA looping and the persistence length of DNA, the *in vivo* results show that looping over short distances is enhanced. For analysis of the data, we present a single equation for the calculation of j_M applicable to all distances. We conclude that chromatin increases the apparent flexibility of DNA ~2-fold, enabling efficient looping over shorter distances than observed *in vitro*.

Results

A single equation to describe DNA looping over all distances

In Figure 1A, the two existing models for the calculation of distance-dependent DNA looping probability in terms of j_M are shown. The Shimada–Yamakawa equation (dotted line) (Shimada and Yamakawa, 1984) and the freely jointed chain model (dashed line) (Rippe *et al.*, 1995) are

shown plotted against distance in base pairs. Each model is valid for only a limited range of distances. To provide a single coherent description of DNA looping, we developed an equation (Equation 2; Materials and methods) that is sufficient alone to calculate j_M for the entire range of distances.

Equation 2 gives a smooth transition between the two existing models for distances between 800 and 1800 bp, and reproduces these two models essentially identically for distances either side of this range (Figure 1A; solid line). For the plots shown in Figure 1A, the persistence length, P , was taken to be 50 nm. Equation 2 is also valid for all other values of P (not shown).

Parameters for DNA twisting, enabling correct torsional alignment of the two sites, were not included in the model. It has been shown theoretically that the dependence of j_M on the helical phasing of the two interacting sites is negligible above 800 bp. Below 800 bp, the j_M values of the model should be taken as averages, where j_M may vary as a function of the relative rotational orientation of the two sites, up to 5-fold for distances of 130–200 bp, and less for longer distances (Shimada and Yamakawa, 1984; Law *et al.*, 1993; Rippe *et al.*, 1995).

The model shown in Figure 1A describes the probability that two DNA sites a certain distance apart on the same molecule will come into direct contact with each other. However, if the two sites are each bound by protein, then an interaction can take place as long as the two sites are brought within a certain distance, r (in nm), which will depend on the size of the protein. In the Shimada–Yamakawa model (up to 2000 bp), it is very difficult to obtain an analytical solution for r values other than zero. Computer simulations have been used to model r (Rippe *et al.*, 1995; Merlitz *et al.*, 1998). In the freely jointed chain model (>2000 bp), the value of r has a negligible effect on j_M if r is between 0 and 20 nm (Rippe *et al.*, 1995). We introduced parameters to model the effect of bound proteins of 10 nm diameter. The parameters were optimized by fitting Equation 3 (Materials and methods) to the published Brownian dynamics simulation data of Merlitz *et al.* (1998) for $r = 10$ nm. As shown in

Figure 1B, Equation 3 gives a good fit to the simulation data of Merlitz *et al.*

In summary, we provide a single equation for protein-mediated DNA looping for any length of DNA, thus incorporating existing theory into a single coherent model.

FLP recombination *in vitro* measures DNA looping

To evaluate whether site-specific recombination can measure DNA looping, a series of FLP recombinase excision substrates was constructed and used in an *in vitro* recombination assay. The substrates have different lengths of DNA between their FRTs, ranging from 74 bp to 15 kb (Figure 2A and B). Purified FLP recombinase was incubated at four different protein concentrations in separate reactions with each substrate at a fixed FRT molarity and DNA concentration. Time points were taken at 2 and 60 min (Figure 2C). In Figure 2D, initial recombination at 2 min is plotted against distance between FRTs at each protein concentration. The curves in Figure 2D strongly resemble that in Figure 1A (Equation 2). Most strikingly, at all four FLP concentrations, the peak in initial recombination was observed between 400 and 700 bp. Although the initial rate of recombination increased with increasing FLP concentration, the peak was not shifted, and no recombination of the 74 bp substrate was observed under any condition.

The fact that the optimum distance for initial recombination is the same at all FLP inputs suggests that FLP measures the intrinsic looping properties of the DNA substrate independently of protein concentration. To evaluate this possibility, we simulated recombination at different FLP concentrations using Equation 4 (Materials and methods), in which DNA looping is described by j_M , and different protein inputs are modelled by varying additional parameters for free protein and incomplete site occupancy. Only these two parameters were varied, thus the calculation of j_M itself was the same for all protein concentrations.

We expect that for any given protein input, the level of initial FLP-mediated excision measured in our assay will be directly proportional to j_M , based on the following reasoning. (i) FLP synapsis occurs by random collision (Beatty *et al.*, 1986); therefore, the probability that two sites a certain distance apart will undergo synapsis is directly proportional to j_M . (ii) The fraction of synaptic complexes that undergo excision is independent of the distance between FRTs. This is a reasonable assumption since the estimated stability of the FLP synaptic complex ($K_{eq} = 9.7 \times 10^8$; Ringrose *et al.*, 1998) is many orders of magnitude higher than the estimated stability of the loop itself for all DNA distances examined ($K_{eq} = 7.6 \times 10^{-5}$ to 2.5×10^{-4} ; Merlitz *et al.*, 1998). This means that once synapsis has occurred via looping, protein-protein interactions should stabilize the complex regardless of any tendency of the DNA itself to unloop. (iii) The reaction is irreversible. Under dilute conditions such as those used here, recombination is exclusively intramolecular, thus reintegration of excised DNA does not occur (Ringrose *et al.*, 1998). Similarly, interference from intermolecular recombination by uneven exchange between FRTs on two substrates was also excluded.

Figure 3A–C shows the curves simulated using Equation 4, superimposed on the measured data points for 12, 6 and 3 nM FLP, respectively. An excellent agreement

between theory and data is seen in all three plots. In particular, the data are consistent with the prediction of the model that no recombination for the 74 bp substrate can take place at any protein concentration (first data point in Figure 3A–C). The predicted optimum distance for recombination is identical in all cases (~500 bp). As seen in Figure 3A–C, this prediction is fully consistent with the observed optimum distance for recombination. Variations in the parameters for site occupancy and free protein concentration affect only the total recombination predicted, and do not shift this peak. For the curve fitting, P was taken to be 50 nm. The fit was worse at all other values of P (not shown). Thus the data are most consistent with a persistence length of 50 nm.

These results demonstrate that FLP-mediated excision gives an accurate measure of DNA looping over the full range of distances studied, independently of protein concentration, thus providing a single method for the quantitative evaluation of DNA looping *in vitro*. Since FLP recombination also works efficiently in living cells, we explored the potential that it could also be used to measure looping *in vivo*.

Experimental strategy to measure looping in active mammalian chromatin

The application of FLP recombination to measure looping in chromatin was evaluated by stable integration of FLP excision substrates in a human cell line. To regulate the onset of recombination so that initial rates of recombination could be estimated, we made use of the inducible recombination system previously described by our laboratory (Logie and Stewart, 1995), in which the activity of an FLP-oestrogen-binding domain (EBD) fusion protein is dependent on oestradiol administration. Initial work showed that inherent variations between stable clones obscured the differences between excision substrates (not shown). Consequently, we developed a relative assay (Figure 4A) where different excision substrates (reporters) were compared with a common reference substrate. The previously described cell line, P1.4 (Logie and Stewart, 1995), contains a stably integrated FLP-EBD expression cassette and a single copy of the pNEO β GAL FLP excision substrate containing two FRTs separated by 1.3 kb. Different excision reporters were stably integrated into P1.4 cells and clones were screened by Southern blotting to select those that retained good induction kinetics after oestradiol administration and contained a single copy of the excision reporter (Figure 4B).

To evaluate the reliability of this assay, the pSVpaZ series of excision reporters (Figure 4A) was used. This series of four constructs contains two directly repeated FRT sites separated by 1.1 kb, and differ only by different FRT half-site configurations. The wild-type FRT (Figure 5A) contains an imperfect 13 bp inverted repeat flanked by a direct repeat. The direct repeat is not required for recombination *in vitro* (Senecoff *et al.*, 1985); however, its relevance *in vivo* has not been determined. Each of the pSVpaZ constructs was transfected into P1.4 cells, and eight or nine independent puromycin-resistant clones, representing different chromosomal integration sites, were selected for each construct. After 10 h oestradiol induction, the pSVpaZ/pNEO β GAL recombination ratio was measured by

Fig. 2. Effect of distance between FRT sites on recombination *in vitro*. (A) Schematic representation of the FRED11 plasmid series of FLP excision substrates, in which directly oriented FRTs (black arrowheads) are separated by varying DNA lengths, from 74 bp to 15 kb. Each FRED11 plasmid was linearized with *NotI* prior to its use in recombination assays. The products of FLP-mediated recombination of all linear FRED11 substrates are a linear molecule of 6.6 kb and a circle of varying size. The unrecombined and recombined linear molecules were detected by Southern hybridization using the probe shown on the figure (grey bar, see also Figure 4A). For constructs in which the distance between FRT sites was 700, 400 or 200 bp, recombination products were digested additionally with *Bam*HI after termination of the recombination reaction to increase the separation between unrecombined and recombined linear fragments, giving a linear recombined fragment of 2.7 kb. (B) Strategy to detect recombination of the FRED11 substrate containing 74 bp between FRTs. *Bgl*II was used for digestion after termination of recombination. The probe used for Southern analysis detects the unrecombined substrate as a fragment of 2.8 kb, and the recombined linear product as a fragment of 6.6 kb. (C) Southern analysis of recombination over different distances at various protein concentrations. Each linearized FRED11 excision substrate (0.05 nM) was incubated with different concentrations of FLP protein as indicated. Aliquots were removed from the reaction at 2 and 60 min. The panels show the 60 min time point data. (D) Quantification of the percentage recombination after 2 min at each protein concentration plotted against distance between FRTs. The percentage recombination was calculated on the basis of PhosphorImager analysis as recombined counts divided by the sum of recombined and unrecombined counts, multiplied by 100.

Southern analysis. Although the absolute rate of recombination varied by up to 4-fold amongst the different clones analysed, the ratio was very similar for all (Figure 5B). Furthermore, when a single pSVpaZ22 clone was analysed after subcloning by dilution to single cells, the ratio was again reliable but similar

moderate variability in the recombination ratio was observed (not shown). These data demonstrate that the assay is robust regardless of the different integration sites of the recombination reporters (33 in total) and define the inherent variability of the assay as up to 2-fold. It should be noted that the absence of position

Fig. 3. Curves for j_{Mrec} fitted to *in vitro* data for initial recombination at 12, 6 and 3 nM FLP. The percentage recombination data of Figure 2D were converted to equivalent j_M units as described in Materials and methods. For each protein concentration, j_{Mrec} was calculated using Equation 4, which includes parameters for incomplete site occupancy (FRT_1 , FRT_2) and free protein (FLP_{free}). In each case, FLP_{free} was specified as approximately equal to input protein concentration (a large excess of protein over FRT sites is present in all experiments), and ($FRT_1 \times FRT_2$) was varied as a single parameter to give the best fit to the data. Curve fitting was done using the Kaleidagraph program (Abelbeck). (A) Open circles: 12 nM FLP, 2 min data points. Solid line: j_{Mrec} , with $FLP_{free} = 1.4 \times 10^{-8}$ M, giving ($FRT_1 \times FRT_2$) = 0.89. (B) Open squares: 6 nM FLP, 2 min data points. Solid line: j_{Mrec} , with $FLP_{free} = 8 \times 10^{-9}$ M, giving ($FRT_1 \times FRT_2$) = 0.76. (C) Open triangles: 3 nM FLP, 2 min data points. Solid line: j_{Mrec} , with $FLP_{free} = 3 \times 10^{-9}$ M, giving ($FRT_1 \times FRT_2$) = 0.27.

effects on recombination observed here is probably due to the fact that all the recombination substrates must be integrated in active chromatin regions permissive for expression of the puromycin resistance gene. The data also show that the third, direct repeat, FRT element is dispensable for FLP recombination in chromatin.

Comparison of recombination over different distances at the same locus

During characterization of pSVpaZ integrants, one clone was isolated in which two copies of pSVpaZ11 had integrated in tandem. The configuration of the double integrant was mapped by Southern analysis (not shown) and is shown in Figure 6A. It contains four tandemly

repeated FRTs. One copy of pSVpaZ11 is incomplete, but both FRTs and the full probe sequence used for Southern analysis are retained. The other copy is complete. The distance between the innermost FRTs is 4.9 kb. FLP recombination can therefore excise DNA between either pair of pSVpaZ11 FRTs 1.1 kb apart, or FRT combinations 4.9, 6.0 or 7.1 kb apart. A time course of recombination was initiated by oestradiol administration. Digestion of genomic DNA with *AccI* enabled recombination of both the 1.1 kb pSVpaZ11 FRTs (sites 1 and 2) and the 1.3 kb pNEO β GAL FRTs to be distinguished (Figure 6B), whilst digestion with *XmnI* enabled detection of recombination events over other distances (Figure 6D).

Quantification of the time course of Figure 6B is plotted in Figure 6C. The rate of recombination at both 1.1 kb sites is the same. The rate of reference recombination at pNEO β GAL is a little slower; however, this difference falls within the range of inherent assay variation noted above. We conclude that for two identical target sites located at the same genomic position, FLP-mediated recombination occurs with equal efficiency, with no preferred order of recombination.

Recombination between the FRTs located 4.9 kb apart, evaluated by *XmnI* digestion of the same DNA samples (Figure 6D), is slower. The most abundant products at early time points were those arising from excisions of 1.1 kb (bands of 18 and 17 kb). Products in which larger excisions had taken place arose at later time points. Furthermore, the 17 kb band containing two FRTs 4.9 kb apart is still substantially unrecombined at the last time point (168 h), whilst the absence in this lane of bands at 19, 18 and 13 kb indicates that all possible excisions of 1.1 kb have taken place. Thus excision of 1.1 kb is more efficient than of 4.9 kb. The data from Figure 6D were quantified and are plotted in Figure 6E as the percentage excision of 4.9 kb or one or more excisions of 1.1 kb. A single exponential was fitted to the data, enabling initial rates to be determined. The 4.9 kb excision event is 2-fold slower than either of those of 1.1 kb. This example indicates that the probability that two sites meet by DNA looping at this locus, as measured by FLP recombination, decreases with the distance between the sites.

Chromatin facilitates DNA looping over short distances

To plot the frequency with which two sites in chromatin meet across a range of distances, the same series of excision substrates used to evaluate looping *in vitro* was integrated into P1.4 cells and relative initial rates of recombination were determined by time course experiments after oestradiol induction (Figure 7A and B). A single exponential was fitted to the data points from each time course and the ratio between reference and reporter at 6 h was calculated from the fitted curves (Figure 7C, open circles).

Remarkably, we observed that recombination of the 74 bp substrate in chromatin was reasonably efficient (Figure 7B), although it had been undetectable *in vitro*. The substrate containing FRTs spaced 200 bp apart was the most efficient in chromatin, again differing from the *in vitro* analysis where greatest efficiency lay at ~500 bp. Thus chromatin appears to enhance looping significantly between sites at short distances.

Fig. 4. Experimental strategy for comparison of FLP-mediated recombination of different reporter targets in mammalian cell culture. **(A)** DNA constructs. P1.4 cells (Logie and Stewart, 1995) carry the FLP/EBD expression vector pHFE1 and a single integrated copy of the recombination reference pNEOβGAL (O’Gorman *et al.*, 1991). A recombination reporter, from either the pSVpaz or FRED series, was stably introduced by selection for puromycin resistance. Candidate clones were screened by Southern analysis before and after oestradiol induction. Clones that retained good oestradiol induction and contained a single copy of the recombination reporter were taken. Promoters are indicated by small arrows, FRT target sites by large arrowheads. *Dra*I restriction sites and the probe (grey bar) relevant to the Southern analyses of **(B)** and Figure 7 are shown. **(B)** An example of a Southern blot used for screening colonies showing induction of recombination by oestradiol. Lane 1, no oestradiol; lane 2, 10 h oestradiol treatment (10^{-7} M). Genomic DNA was digested with *Dra*I, permitting the four unrecombined and recombined bands to be distinguished as indicated.

Fig. 5. Reliability of the *in vivo* comparative recombination strategy using pSVpaz recombination reporters. **(A)** The wild-type FRT. Inverted repeat elements are shown as horizontal arrows. The non-palindromic spacer is indicated by an open box. Elements a and b differ by a single mismatch at the second position of the repeat element (indicated in bold). **(B)** Relative recombination ratios of different target site configurations in cell culture. Eight or nine independent P1.4 clones containing single copy integrants of pSVpaz constructs were evaluated for oestradiol-induced recombination after 10 h. The various configurations of FRT half-sites in pNEOβGAL and pSVpaz are illustrated. pNEOβGAL contains one minimal and one full FRT (Snaith *et al.*, 1996). pSVpaz11 contains two minimal FRTs and pSVpaz22 contains two full FRTs. The number (*n*) of independent clones analysed for each construct, representing different pSVpaz integration sites, is indicated alongside the mean and standard deviation of the ratio of pSVpaz/pNEO recombination.

To compare the chromatin data with theoretical predictions for naked linear DNA, the model of Equation 3 ($r = 10$ nm) is shown superimposed on the data in Figure 7C (dotted line). j_M values were scaled to recombination ratios in chromatin as described in the figure legend. Parameters for incomplete site occupancy and free protein were

not included in this analysis because recombination is measured as a ratio, and both reporter and reference substrates should be affected equally by these parameters. It is important to note that both scaling, and variation of parameters for site occupancy and free protein have the effect of increasing or reducing j_M , but do not shift the optimum distance for recombination, nor enable looping over 74 bp.

The curve given by Equation 3 gives a poor fit to the *in vivo* data for distances of <2 kb, predicting no recombination of 74 bp and little at 200 bp. For distances between 400 bp and 2 kb, more recombination is predicted than observed. The ability to form shorter loops than predicted theoretically and the shorter optimum distance observed *in vivo* suggest that DNA in chromatin is bent more easily than naked DNA at short distances. To test this idea, the persistence length, P , was varied in Equation 3 ($r = 10$ nm) to optimize the fit of the model to the data. To emphasize the fact that P itself is an inherent property of DNA, and that the parameter we are examining is the apparent flexibility, we call this parameter P_{app} . The solid line in Figure 7C shows the best fit obtained, for which $P_{app} = 27$ nm, showing that an increase in apparent flexibility of ~ 2 -fold greatly improves the fit of the model to the data at distances up to 2 kb. In particular, recombination of the 74 bp substrate becomes possible and the optimum distance for recombination is shifted to 200 bp. Thus our data are consistent with an increased apparent flexibility of chromatin-packaged DNA at short distances.

Discussion

We present a single equation (Equation 2) that incorporates and extends existing models for DNA looping. In particular, a protein of 10 nm diameter can be modelled by

A pNEO β GAL **pSVpaz11 double integrant** **B** **C** **D** **E**

Fig. 6. Recombination of a tandemly integrated recombination reporter in P1.4 cells. **(A)** Strategy for detection of distinct recombination events. In the double integrant, the two pairs of pSVpaZ11 FRTs (arrowheads) are 4.9 kb apart, and are labelled 1 and 2. The restriction sites and probe hybridization sites used for Southern analyses are shown. **(B)** Southern analysis of a recombination time course after oestradiol induction. Recombinations at site 1, site 2 and pNEO β GAL were distinguished by digestion with *AccI* and are indicated at the side of the panel. **(C)** Quantification of **(B)** for recombination at pNEO (▲), site 1 (●) or site 2 (○). The percentage recombination was calculated from PhosphorImager quantifications for each target site as the counts in the recombined band divided by the sum of the counts in the recombined and its corresponding unrecombined band, multiplied by 100. For each data set, the best fit of the single exponential curve described by the equation $R = F(1 - e^{-kt})$ was obtained using Kaleidagraph (Abelbeck Software). R = percentage recombination at time t ; F = final recombination. The value of F was set at 100%. **(D)** Southern analysis of the genomic DNA samples from the time course experiment shown in **(B)** were digested with *XmnI*. The various recombination events observed are indicated at the side of the panel. **(E)** Quantification of 1.1 and 4.9 kb recombination events. The Southern blot of **(D)** was quantified by PhosphorImager analysis. The percentage of double integrants in which one or more excisions of 1.1 kb had taken place was calculated for each lane as the sum of counts in the 18, 17, 13 and 12 kb bands, divided by the total counts in the lane (excluding bands for pNEO), multiplied by 100. The percentage of double integrants in which an excision of 4.9 kb had taken place was calculated similarly on the basis of the 12 and 13 kb bands. For each data set, the best fit of the single exponential curve was obtained as described in **(C)** above, except that the value of F was allowed to vary.

Fig. 7. Effect of distance between FRT sites on recombination in chromatin. (A) Example of a time course experiment to determine the ratio of reporter to reference recombination. A stable P1.4 clone carrying the FRED11 1.1 reporter was induced with oestradiol for various times before harvesting DNA for *Dra*I digestion and Southern analysis (see Figure 4A). The expected fragments for unrecombined and recombined pNEO β GAL and FRED11 1.1 constructs are indicated. (B) As for (A) except that a clone containing the 74 bp FRED11 0074 recombination reporter is shown. DNA was digested with *Acc*I and *Bgl*II to detect recombination products of the sizes indicated. (C) Plot of initial reporter/reference recombination ratios against distance. For each FRED11 recombination reporter construct, stable cell lines were established and time courses of recombination as shown in (A) and (B) were performed. Recombination of both reporter and reference was quantified by PhosphorImager analysis. For each time course experiment, a single exponential curve (as for Figure 6E) was fitted separately to the data for FRED and pNEO, and the FRED/pNEO ratio at 6 h is plotted ($\circ$). Dotted line: j_M , the distance-dependent looping probability for naked linear DNA *in vitro*, with persistence length $P = 50$ nm, calculated using Equation 3 ($r = 10$ nm). All j_M values were multiplied by 2.67×10^7 for scaling to FRED/pNEO values. Solid line: j_M for DNA in chromatin, fitted to data using Equation 3 with $P = 27$ nm, $r = 10$ nm. All j_M values were multiplied by 4.2×10^6 to give the equivalent FRED/pNEO scale.

inclusion of r , the proximity within which two sites must be to communicate (Equation 3). Previously, in the absence of an analytical solution for r at short distances, the effect of bound proteins has been determined by simulation experiments (Rippe *et al.*, 1995; Klenin *et al.*, 1998; Merlitz *et al.*, 1998). We used data from such simulations to determine the parameters for r . With more simulation data for other r values, a full description of r could be included in Equation 3. Similarly, parameters to include the effects of DNA bends on looping, based on computer simulations (Merlitz *et al.*, 1998), can be added (not shown).

The simplicity of Equation 2 offers insight into the factors that govern DNA flexibility. For a persistence length of 50 nm, the third term in Equation 2 is equal to 1. The two other terms are the freely jointed chain of Equation 1 and an exponential term that reduces the value of the freely jointed chain term at short distances (Figure 1A). Thus the exponential term may have physical meaning, describing the increase in the constraining effects of DNA stiffness on the ability of a freely jointed chain to loop as its length decreases.

Using FLP recombination as a measure of DNA looping

in vitro, we show that the best fit of the model of Equation 3 to the data is given by a persistence length of 50 nm, agreeing with previous measurements (Hagerman, 1988). Inclusion of a bound protein in the model was necessary for an optimal fit. Parameters for excluded volume effects, which can reduce the apparent flexibility of chains longer than 2.7 kb (Rivetti, 1996), were not included in the model, since a good fit was obtained without them.

Using FLP as a measure of looping *in vivo*, we show that DNA in chromatin is able to form smaller loops and has a shorter optimal distance for looping (200 bp) than *in vitro* (500 bp). The effects of this increased flexibility are most striking at distances of <2 kb, and can be described in our model by reducing the apparent persistence length to 27 nm.

There are several potential limitations that could complicate the interpretation of these experiments. First, the fact that an FLP recombinase fusion protein was used in the cell culture experiments raises the possibility that the looping observed over shorter distances in chromatin is due to the larger diameter of the protein. The ligand-binding domain increases the size of FLP from 423 to

767 amino acids. Within this size range, variations in the value of r in Equation 3 according to Merlitz *et al.* (1998) do not shift the peak at which optimum looping occurs, nor do they enable looping of a 74 bp fragment. Thus the increased size of the protein cannot account for the *in vivo* data.

Secondly, much attention has been given in the literature to the idea that sequence-specific bending may be necessary to enable looping *in vivo* over the short distances observed between eukaryotic upstream elements and their promoters (Rippe *et al.*, 1995; Schatz and Langowski, 1997; Merlitz *et al.*, 1998). The requirement for an inherent sequence-specific bend to enable the 74 and 200 bp substrates to loop in our assay can be ruled out because we have compared identical sequences *in vitro* and *in vivo*, with different results. However, we cannot rule out the possibility that our results *in vivo* are affected by bending induced by unknown sequence-specific binding protein(s). This is very unlikely since recombination *in vivo* of the 200, 400, 700, 1100 and 1900 bp substrates is reduced progressively with increasing distance. Furthermore, the constructs used were not generated systematically. This suggests a relationship between distance and looping that is not subject to sequence-specific effects. The relative rates of recombination over 1.1 and 4.9 kb observed from tandem integration of a different recombination substrate, pSVpaZ22, lend further support to this interpretation.

A third complication in the interpretation of our results could be the presence of positioned nucleosomes (van Holde, 1988). If in all substrates of >200 bp, one FRT was occluded by a nucleosome whose position was determined by the DNA sequence, then a different set of substrates could give a different result. The same reasons given above to discount a significant impact of protein-induced sequence-specific bending also apply to discount FRT occlusion by a positioned nucleosome. We also note that <5% of bulk genomic DNA sequences contribute to their own packaging at the nucleosome level (Lowary and Widom, 1997).

Thus we reason that our *in vivo* results reflect the inherent distance-dependent looping dynamics of DNA packaged in transcriptionally active chromatin. The two effects of chromatin packaging most important to this study are DNA compaction and binding site occlusion (Hildebrandt and Cozzarelli, 1995). DNA compaction in chromatin occurs on at least two levels: wrapping of DNA in nucleosomes, and higher order packaging. This condensation causes distant DNA sites to be brought together. This may occur statically, due to spatial juxtaposition of sites, but may also be a dynamic process, involving transient meeting of sites upon changes in chromatin organization. In both cases, DNA compaction increases the probability that two sites will meet. On the other hand, the presence of nucleosomes and their higher order organization leads to the occlusion of binding sites, reducing the probability that they will be occupied by protein. Again, site occlusion may be static or dynamic. For recombination in chromatin, the effects of DNA compaction and binding site occlusion are thus in opposition, and their relative contributions to the efficiency of the reaction will vary at each of the distances studied.

For the 74 bp substrate, the most striking observation is that it can be recombined *in vivo*, but not *in vitro*. The

in vitro result is consistent with the theoretical prediction that naked DNA cannot form a loop of 74 bp. Thus we conclude that *in vivo*, the two FRTs are brought together by chromatin-mediated compaction of DNA. Given that 146 bp of DNA wrap twice around one nucleosome (Luger *et al.*, 1997), and that the linker DNA may be variable in length (van Holde, 1988), there are four possible arrangements of two FRTs 74 bp apart. First, both FRTs could be in the same nucleosome, in which case they would lie side by side on the nucleosome surface, since there are 73 bp per turn of DNA around the nucleosome (Luger *et al.*, 1997). Secondly, one FRT could be in the linker, and the other in the nucleosome. Thirdly, both FRTs could be in one linker. Finally, the two FRTs could be on either side of a linker and thus at the edges of adjacent nucleosomes. It has been suggested that nucleosomes, by constraining the ends of linker DNA, may be enabled to adopt a supercoiled configuration (van Holde, 1988; Rippe *et al.*, 1995). Computer simulations have shown that the juxtaposition probability in naked supercoiled DNA is two orders of magnitude higher than in relaxed DNA (Vologodskii and Cozzarelli, 1996; Jian *et al.*, 1998). Thus, supercoiling of a trapped domain may account for the recombination of the 74 bp substrate in chromatin. However, experimental observation of supercoiling in trapped linkers has not been reported. Instead, there is now strong evidence from independent approaches, both *in vitro* and *in vivo*, that nucleosomes are arranged in a zig-zag pattern, with extended linkers, and that this arrangement exists in both the compact and the extended fibre (Bednar *et al.*, 1998; Lueba *et al.*, 1998; Rydberg *et al.*, 1998). In such a structure, at all levels of compaction, two FRTs spaced 74 bp apart will only be brought closer than in linear DNA if they are in the same nucleosome. In all other arrangements, the 74 bp between the FRTs is fully or almost fully extended. Thus we reason that the most likely pathway for recombination of the 74 bp substrate is synapsis of two FRTs that lie side by side on the same nucleosome.

Two non-exclusive mechanisms for juxtaposition on the nucleosome surface of two FRTs 74 bp apart can be envisaged. If chromatin is static once assembled and nucleosomes are positioned randomly but do not move around, then two distinct populations of substrates should exist: those in which the FRTs are juxtaposed and those in which they are not. If, on the other hand, nucleosomes in active chromatin are dynamic and able to slide without dissociating from DNA, then the two FRTs may be juxtaposed in all substrates but only transiently (Pazin *et al.*, 1997; Varga-Weisz and Becker, 1998). Our results do not enable these two mechanisms to be distinguished. In both cases, if FLP synapses two FRTs on the nucleosome surface, then partial recombination of the 74 bp substrate would be expected.

Synapsis on the nucleosome surface requires that FLP can bind two FRTs on nucleosomal DNA, despite site occlusion. There is strong evidence from *in vitro* studies that nucleosomes are indeed dynamic structures, continually transiently exposing stretches of their DNA, and that proteins can gain access to nucleosomal binding sites without histones leaving DNA (Adams and Workman, 1995; Polach and Widom, 1995, 1996; Studitsky *et al.*, 1995, 1997; Protacio and Widom, 1996; Juan *et al.*, 1997).

Studies to date have concentrated mainly on binding of a single protein, binding of two proteins that do not interact with each other or bypassing of nucleosomes by RNA polymerases. If, as we expect, FLP binds and synapses whilst the nucleosome is still present, then our data provide *in vivo* evidence of a new class of interaction: looping on the nucleosome surface.

The 200 bp substrate recombines most efficiently of all distances tested, and six times more efficiently than the 74 bp substrate. A quantitative study of DNA compaction in the chromatin fibre in living nuclei has shown that two points which are 185 bp apart are found in juxtaposition about three times less frequently than for 78 bp. Sites separated by 185 bp come close together only on linker DNA exiting and entering a nucleosome, whilst sites separated by 78 bp are juxtaposed all around the nucleosome core (Rydberg *et al.*, 1998). As discussed above, this juxtaposition may be either static or dynamic. Since compaction is higher for 78 bp than for 200 bp, we interpret the ease with which the 200 bp substrate can recombine as the result of reduced occlusion. In this substrate, the two sites may be in adjacent linkers, or on adjacent nucleosomes, but will never be on the same nucleosome. This means the pair of sites will be more available than those in the 74 bp substrate. We conclude that the distance of 200 bp represents the optimum combination of compaction and availability for recombination. The 400 bp substrate, since it is a multiple of 200, should be subject to the same occlusion as the 200 bp substrate. The reduction of recombination efficiency in this substrate is thus probably due to reduced compaction.

Recombination of the 74, 200 and 400 bp substrates involves interactions at the level of the single nucleosome or two adjacent nucleosomes, and thus we expect the effects of occlusion and compaction to be non-uniform over this range, having discrete peaks in recombination for favoured distances. Experiments with substrates in which FRTs are at intermediate distances would be necessary to test this idea. Longer distances cover several nucleosomes, and we expect that as DNA length increases, the effects of compaction and occlusion will be evened out (Rydberg *et al.*, 1998). Above a certain length, recombination efficiency should be determined by physical distance alone and the chromatin fibre should behave as a uniform polymer. The fact that the polymer-based model of Equation 3 gives a reasonable fit to the *in vivo* data above 2 kb (Figure 7C) is consistent with this prediction. We point out, however, that because of the scaling procedure used, quantitative conclusions about the persistence length of the chromatin fibre cannot be drawn for this range of distances. For short distances, on the other hand, the apparent persistence length, P_{app} , of 27 nm (Figure 7C) has quantitative meaning because it shifts the peak at which maximum recombination occurs. In this size range, it should be possible to modify the model of Equation 3 to describe specifically DNA in chromatin, by dissecting the parameter P_{app} into more realistic distance-dependent terms for compaction and occlusion. In conclusion, we propose that short- and long-range looping interactions in chromatin occur by distinct mechanisms, and are governed by different sets of constraints.

We have used FLP to measure looping dynamics in transcriptionally active chromatin. The sensitivity of FLP

to both occlusion and compaction raises the possibility that it could be used to detect transitions between different chromatin states in living systems more effectively than assays based on protein access alone (Schlossherr *et al.*, 1994; Wallrath and Elgin, 1995; McCall and Bender, 1996; Boivin and Dura, 1998).

Materials and methods

Plasmids

The FLP/EBD expression plasmid pHFE1 and the excision substrate pNEO β GAL are described elsewhere (O'Gorman *et al.*, 1991; Logie and Stewart, 1995). The pSVpaZ series of excision substrates containing different configurations of FRT target sites (Figure 5) were constructed by ligation of appropriate oligonucleotides into pSVpaZ11 (Buchholz *et al.*, 1996).

The FRED11 series of excision substrates, containing different lengths of DNA between the FRT target sites, were constructed from pSVpaZ11 as follows. FRED11 0074, containing 74 bp between the two FRT sites (measured from the centres of the spacers), was made by removing an *AvrII*–*HindIII* fragment from pSVpaZ11 containing the 5' FRT, leaving the SV40 promoter and the puromycin resistance gene intact (see Figure 4A). An oligonucleotide containing an FRT identical to that removed, and a *BglII* site, was ligated into this plasmid downstream of its remaining FRT. The resulting plasmid thus carries two FRTs in direct orientation, flanked on the 5' side by the puromycin resistance gene driven by the SV40 promoter (see Figure 4A).

FRED11 15 was made by ligating 15 kb of *BglII*-digested yeast DNA into the unique *BglII* site of FRED11 0074. Sequence analysis of the 3' and 5' ends of the yeast insert enabled identification of the entire sequence from the EMBL database as the 15 kb *BglII* fragment of chromosome X containing the YJL223c locus.

FRED11 9.9, containing 9.9 kb between the two FRTs, was made from FRED11 15 by removal of a 5.1 kb *EcoNI* fragment. FRED11 7.9 was made from FRED11 15 by removal of a 7.1 kb *XhoI* fragment. FRED11 4.5 was made from FRED11 15 by removal of a 10.5 kb *EcoNI*–*SnaBI* fragment. FRED11 2.9 was made from FRED 11 15 by removal of a 12.1 kb *SnaBI*–*BstBI* fragment. FRED11 1.9 was made from FRED11 7.9 by removal of an *Apal*–*AvrII* fragment. FRED11 1.1 was made by exonuclease III digestion of *Apal*–*AvrII*-cut FRED11 7.9. FRED11 07, containing 0.7 kb between the two FRTs, was made from FRED11 1.9 by removal of an *EcoNI*–*HindIII* fragment. FRED11 04 was made from FRED11 1.9 by removal of a *BstBI* fragment. FRED11 02 was made from FRED11 1.9 by removal of a *BglII*–*Asp718* fragment.

Cell culture

Culture and stable transfection of the P1.4 cell line, which contains stably integrated pHFE1 and pNEO β GAL, have been described (Logie and Stewart, 1995). Derivatives of P1.4, containing randomly integrated pSVpaZ or FRED constructs, were generated by transfecting cells with *NotI*-linearized pSVpaZ or FRED plasmids followed by selection of stable colonies with 1 μ g/ml puromycin (Sigma). Individual clones were screened by Southern blotting before and after 10 h of oestradiol induction of recombination. Clones that did not show an intact single copy of the recombination reporter or which showed <10% recombination of the pNEO β GAL reference (indicating a loss of FLP recombinase inducibility) were excluded. Recombination was induced by culturing cells in 10^{-7} M oestradiol. All cell culture included hygromycin, which may enhance the transcriptional activity of the FLP/EBD expression cassette. In the case of the pSVpaZ plasmid series, several independent clones were analysed for recombination activity. For the FRED series, one or two clones were analysed for each construct. Genomic DNA was extracted, digested and subjected to Southern analysis as previously described (Logie and Stewart, 1995).

In vitro recombination assay

The FLP protein preparation used for *in vitro* recombination experiments was a generous gift from Michael Cox. The preparation is >95% pure as judged by SDS-PAGE, and was purified as described (Meyer-Leon *et al.*, 1987). Recombination assays were carried out using the FRED series of excision substrates linearized with *NotI*. Reactions contained 0.05 nM of each FRED substrate, and varying amounts of FLP protein. To compensate for the different sizes of the FRED plasmids, the total DNA in each reaction was made up to 0.7 ng/ μ l using λ DNA digested

with *Hind*III. Reactions were incubated at 25°C in 25 mM TAPS pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 10% glycerol, 5% polyethylene glycol (PEG) 8000 and 2.5 mg/ml bovine serum albumin (BSA). Reactions were terminated and electrophoresed as described (Buchholz *et al.*, 1996). Recombination products were detected by Southern hybridization and quantified by PhosphorImager analysis.

Mathematical methods

In all equations, the distance-dependent looping probability is expressed in terms of j_M , which is the molar concentration of one site with respect to the other (Rippe *et al.*, 1995).

We took as a starting point Equation 3 of Rippe *et al.* (1995) describing the freely jointed chain model, valid for $bp = 2000$ and above. The parameter P (= persistence length, in nm) was introduced on the basis of Equation 2 of the same reference, giving:

$$j_M(bp, P) = [4P/(10^4 \times bp)]^{3/2} \quad (1)$$

(plotted for $P = 50$ in Figure 1A, dashed line).

To render the model valid for all DNA distances, terms were introduced whose effect is to reduce the value of Equation 1 at shorter distances, such that the model of Shimada and Yamakawa (1984) is reproduced exactly. For this purpose, Equation 50 of Shimada and Yamakawa (1984), giving ring closure probability without parameters for torsional alignment of sites, was converted to j_M units according to Table I of Hagerman and Ramadevi (1990) (plotted for $P = 50$ in Figure 1A, dotted line).

The full model for all DNA lengths is given by:

$$j_M(bp, P) = [4P/(10^4 \times bp)]^{3/2} \times \exp(-460P^2/6.25bp^2) \times (1.25 \times 10^5/P^3) \quad (2)$$

(plotted for $P = 50$ in Figure 1A, solid line).

The proximity at which the DNA ends must be for an interaction to occur is expressed by the parameter r , in nm. Equation 2 is valid for $r = 0$. Parameters enabling the value of r to be modified, to model the effect of bound proteins, were introduced into the exponential term of Equation 2, and values for $r = 10$ nm were determined by optimizing the curve to fit Brownian dynamics simulation data for j_M (Rippe *et al.*, 1995; Merlitz *et al.*, 1998) (Figure 1B).

$$j_M(bp, P, 10) = [4P/(10^4 \times bp)]^{3/2} \times \exp[-(460 + 50)P^2/(6.25bp^2 + 50P^2)] \times (1.25 \times 10^5/P^3) \quad (3)$$

To fit the model to *in vitro* data (Figure 3), percentage recombination was converted into j_M units as follows. Maximum recombination measured at 2 min at 12 nM protein input (R_{max} , in M) was taken as equivalent to the maximum value of j_M given by Equation 2 (j_{Mmax}). All measured levels of recombination were then multiplied by j_{Mmax}/R_{max} . Equation 3 is valid only for conditions in which the two FRTs are always fully occupied and in which the molar concentration of free protein is much smaller than j_M (Rippe *et al.*, 1995). However, in the experiment of Figure 2, the molar concentrations of FLP were comparable with j_M . In addition, due to the low specific binding affinity of FLP for the FRT, even in the presence of excess FLP, only a proportion of FRTs are occupied at equilibrium under conditions similar to those used here (Ringrose *et al.*, 1998). Therefore, to take account of these conditions, the probability of a successful recombination event, j_{Mrec} , was calculated according to Equation 4 of Rippe *et al.* (1995).

$$j_{Mrec} = FRT_1 \times FRT_2 \times j_M + FLP_{free} \quad (4)$$

For fitting Equation 4 to *in vitro* recombination data, j_M was given by Equation 3. (FLP_{free}) was taken as approximately equal to the input protein concentration (a large excess of protein over FRT sites is present in all reactions), and ($FRT_1 \times FRT_2$) was determined for each protein input separately by fitting the curve to the data. The values of ($FRT_1 \times FRT_2$) determined from curve fitting were approximately proportional to the square of FLP concentration up to 6 nM. Above this concentration, the curve levelled off as saturation was approached. Thus the occupancy of each single FRT [square root of ($FRT_1 \times FRT_2$)] increased linearly with FLP concentration, until the system was saturated (not shown).

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