Localization of curved DNA and its association with nucleosome phasing in the promoter region of the human estrogen receptor α gene

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Abstract We determined DNA bend sites in the promoter region of the human estrogen receptor (ER) gene by the circular permutation assay. A total of five sites (ERB−4 to −1, and ERB+1) mapped in the 3 kb region showed an average distance of 688 bp. Most of the sites were accompanied by short poly(dA)·poly(dT) tracts including the potential bend core sequence $A_2N_8A_2N_8A_2$ (A/A/A). Fine mapping of the ERB−2 site indicated that this A/A/A and the 20 bp immediate flanking sequence containing one half of the estrogen response element were the sites of DNA curvature. All of the experimentally mapped bend sites corresponded to the positions of DNA curvature as well as to nucleosomes predicted by computer analysis. In vitro nucleosome mapping at ERB−2 revealed that the bend center was located 10–30 bp from the experimental and predicted nucleosome dyad axes.

Key words: DNA bend site; Curvature; Nucleosome mapping

1. Introduction

The estrogen receptor (ER) is indispensable for estradiol-dependent regulation of developmental, endocrine and behavioral functions [1] and is a member of a large family of steroid-thyroid hormone receptors that mediate signals from estradiol and other ligands to the genes in the signal transduction pathway [2,3]. The gene that encodes this receptor is located on chromosome 6, and has eight exons encompassing more than 140 kb [4]. One of the unique features of this gene is the presence of an alternative promoter, P0, located 2 kb upstream of the canonical promoter, P1 [5,6]. The transcripts from the P0 promoter have an additional exon (exon I′) 96 bp long and a 1860 bp intron (intron I′). Differential utilization between these promoters was observed in human primary osteoblasts and several cell lines [7]. Exon I′ contains an open reading frame for a peptide of 18 amino acid residues, which is of unknown function but may confer tissue specificity of transcription. Moreover, several DNA sequence elements including the estrogen response element (ERE) and a higher order of chromatin structure revealed by DNase I-hypersensitive sites have been suggested to be essential elements for this tissue specificity [8].

A nearly periodic distribution of curved DNA sites was originally found in the human ε-globin gene region [9]. These sites were revealed by the circular permutation assay where restriction fragments exhibited 5–20% retardation relative to normal migration in polyacrylamide gels. Further studies indicated that these sites (i) were repeated at intervals of approximately 680 bp on average, (ii) were conserved in the molecular evolution of genomic DNA, and (iii) showed disturbances of the periodicity at exons, junctions of genome rearrangements, and other functional regions [10–12]. Their average interval, which roughly corresponded to the length of four nucleosomes, and their universal presence in the functionally inert intergenic region suggested that these bend sites may function as signals for chromatin folding or nucleosome phasing. This was strengthened by the presence of disturbances of the periodicity at the DNase I-hypersensitive sites in the human β-globin locus control region (unpublished results), which are believed to be open chromatin regions and to act as insulators for transcription and replication. These sites, furthermore, determine the nucleosomal phases by having a higher affinity to core histones, and thus should play a key role in initiation of nucleosome formation [12]. A detailed experimental and computational analysis of the location of curved DNA and of the nucleosomes in the promoter region of the human ER gene is presented, confirming and complementing the earlier conclusion on the functional importance of the nearly periodic bend sites in the eukaryotic genomic DNA.

2. Materials and methods

2.1. The circular permutation assay

The protocol for the circular permutation assay was described previously [9]. To map DNA bend sites in the promoter region of the ER gene, the region was divided into eight subclones of 500–800 bp in length which contained tandem duplicates of the cloned regions. The regions were divided further if more than one site was detected. After digestion of the cloned DNA with restriction enzymes having a unique site in the cloned region, the DNA fragments generated were electrophoresed on 8% polyacrylamide gels (mono:bis = 29:1) in 45 mM Tris-borate (pH 8.0), 1 mM EDTA buffer at either 4°C or 55°C. The gels were stained with ethidium bromide after electrophoresis. The regions used for the assay were: −2708 to −2100 (pTH), −2541 to −2084 (pPV), −2100 to −1461 (pHT), −1546 to −725 (pMS), −1238 to −725 (pPS), −725 to −29 (pSC), −235 to +284 (pSAU1) and +79 to 478 (pSM), relative to the cap site P1. These regions were cloned into pBluescript SK^- vector at the *Cla*I (for *Taq*I and *Hin*PI fragments), *Pst*I (for *Pst*I fragments), *Bam*HI (for *Bst*YI fragments) or *Eco*RV sites (for blunt end fragments).

2.2. Curvature assay with oligonucleotides

Twenty-mer oligonucleotides TTCACCTATTTTTTGTCTCC (for T1), TTTAATTCAGTTAAAAACTT (for T2), TCGTGACCTGAGGTTATGTT (for T3A), AGGTTATGTTTGGTATGAAA (for T3AB), TGGTATGAAAAGGTCACATT (for T3B), TTA-

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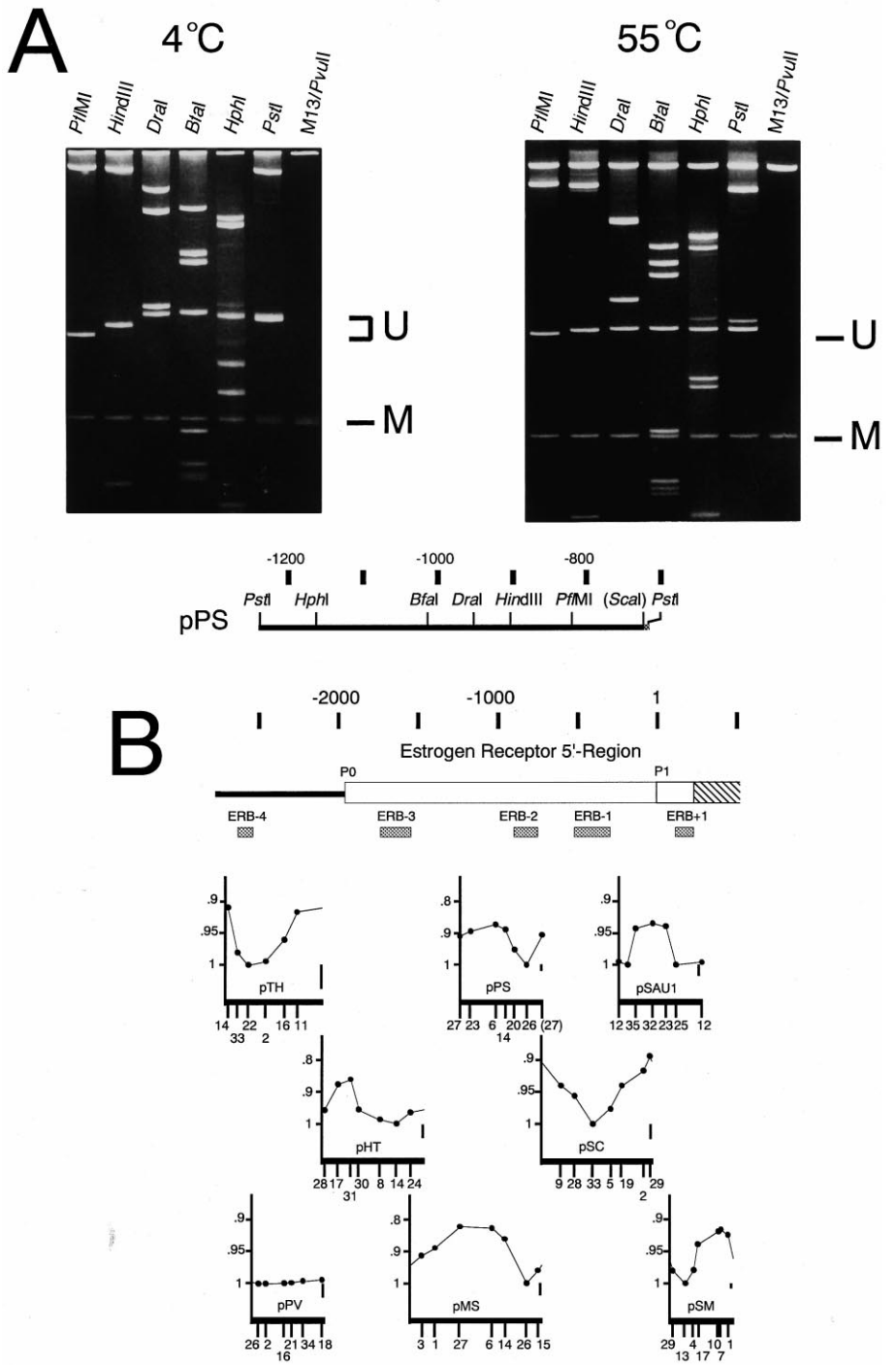

Fig. 1. Mapping of DNA bend sites in the promoter region of the ER gene. A: The plasmid pPS containing the region of nucleotides −1238 (*Pst*I) to −725 (*Sca*I) relative to the canonical cap site (P1) was used for the circular permutation assay [22]. A *Pst*I linker was attached to the *Sca*I end of the fragment, abolishing the *Sca*I site (in parentheses) and creating a new *Pst*I site at the right end. The plasmid DNA digested with the restriction enzymes shown above was electrophoresed through the polyacrylamide gels at 4°C (left) or 55°C (right). U: unit length fragment; M: M13mp18 digested with *Pvu*II as a marker. B: Summary of mapping. Each panel shows the relative rate of migration in the gels on the vertical axis and the positions of the restriction enzymes on the horizontal axis. The bend sites (ERB−4 to ERB−1 and ERB+1) are indicated as the shadowed boxes. Each panel contains the average thickness of the bands as a vertical bar. The canonical cap site (P1) and the alternative cap site (P0) are shown. The restriction enzymes used are: 1, *Acc*I; 2, *Alw*NI; 3, *Ase*I; 4, *Ava*II; 5, *Bam*HI; 6, *Bfa*I; 7, *Bsa*HI; 8, *Bsm*AI; 9, *Bsm*I; 10, *Bsr*GI; 11, *Bst*71I; 12, *Bst*YI; 13, *Dde*I; 14, *Dra*I; 15, *Eco*RI; 16, *Eco*RV; 17, *Fok*I; 18, *Hae*II; 19, *Hha*I; 20, *Hin*dIII; 21, *Hin*fI; 22, *Hpa*I; 23, *Hph*I; 24, *Msc*I; 25, *Nsp*I; 26, *Pfl*MI; 27, *Pst*I; 28, *Pvu*II; 29, *Sac*I; 30, *Sau*3AI; 31, *Sau*96I; 32, *Sca*I; 33, *Sna*BI; 34, *Sty*I; 35, *Taq*I. The restriction site derived from a linker is shown in parentheses.

TATTCAGTTTTCTGAAG (for T3) and TTTTGGTTGCATAACCAACC (for T3C) were annealed with the respective complementary oligonucleotides, which were designed to create two-base 5′-protruding ends, at a final concentration of 0.2 mg/ml. The size standard $A_{20}+T_{20}$ was prepared by annealing $(dA)_{20}$ with $(dT)_{20}$. The annealed duplexes (50 μl) were then incubated with 10 units of T4 polynucleotide kinase in the presence of 1 mM ATP at 37°C for 30 min, followed by reaction with T4 DNA ligase (400 units) at 4°C overnight. The ligation products were resolved by electrophoresis through an 8% polyacrylamide (29:1 = mono:bis) gel in 45 mM Tris-borate, 1 mM EDTA buffer at 4°C for 23 h.

2.3. Analysis of the nucleosome positions

Nucleosome core particles were prepared as described [12]. DNA samples were mixed with the purified nucleosome core particles in 100 μl of TEP (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM PMSF) supplemented with 2 M NaCl and dialyzed first against 1 l of TEP with 0.4 M NaCl for 3 h at 4°C, and then against 1 l of TEP with 16 mM NaCl overnight at 4°C. For restriction analysis of nucleosomal phases, 10 μg aliquots of core particles were mixed with 20 μg of plasmid DNA (pBluescript or pPS) linearized with *Xmn*I. Reconstituted core particles were digested with 20 U/ml micrococcal nuclease for 40 min on ice followed by incubation at 37°C for 3 min [13]. The ∼146 bp fragments protected by core particles were twice recovered from a 6% polyacrylamide gel (19:1 = mono:bis), treated with bacterial alkaline phosphatase and then labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The labeled fragments were digested with restriction enzymes and, after ethanol precipitation, resolved by 6% polyacrylamide-7 M urea gels under denaturing conditions.

2.4. Computer analysis for curvature and nucleosome mapping

The expected positions of DNA curvature and nucleosome dyad axes were calculated with the software TRIF 1.00 (ftp://sgjs1.weizmann.ac.il/usr/users/Curvature) based on the distribution of dinucleotides TT and AA for curvature mapping or the nucleosome matrix for nucleosome mapping as described previously [14–16]. One curvature unit roughly corresponds to 90°/20 bases.

3. Results

3.1. DNA bend sites in the promoter region of the ER gene

We first mapped the DNA bend sites in the promoter region of the ER gene by the circular permutation assay. Fig. 1A shows the results of the assay using the plasmid pPS containing the region from −1238 (*Pst*I site) to −725 (*Sca*I site) relative to the cap site of the ER gene. After digesting the plasmid with the restriction enzymes as indicated, the samples were electrophoresed on 8% polyacrylamide gels at 4°C (left) or 55°C (right). The presence of curved DNA caused retardation of the 513 bp fragment. The effect was maximal when the structure was located close to the center of the fragment (*Bfa*I fragment) and minimal when the structure was located close to either end (*Pfl*MI). Thus, from the relative migration of the restriction fragments, the bend center was mapped between the *Hin*dIII and *Sca*I sites, close to the *Pfl*MI site. If there had been no bend sites in the cloned fragments, all fragments would have shown identical mobility (see pPV below). Using this strategy, we mapped a total of five sites (ERB−4 to ERB−1 and ERB+1) in the 3 kb region (Fig. 1B) which showed an average interval of 688 ± 210 (S.D.) bp. The distances between the centers of the bend sites were 948 ± 146 (half of the area for both sites) bp between ERB−4 and ERB−3, 826 ± 188 bp between ERB−3 and ERB−2, 411 ± 208 bp between ERB−2 and ERB−1, and 567 ± 183 bp between ERB−1 and ERB+1. Although the distances between ERB−3 and ERB−2 and between ERB−1 and ERB+1 were close to the average in this 3 kb region and the other loci, the other two distances were not. The presence of the second cap site (P0) at 1955 bp upstream from the canonical site (P1) seemed to have caused this disturbance.

The nucleotide sequences of the bend sites indicated that they were generally A+T-rich and contained multiple A or T tracts of three nucleotides or more (data not shown). Many of the tracts are distributed periodically close to DNA helical repeat. Note that three (ERB−3, −2 and −1) of the five sites contained the potential bend core sequence $A_2N_8A_2N_8A_2$ (A/A/A) or $T_2N_8T_2N_8T_2$ (T/T/T) [10,11]. These sequences were previously shown to be the actual bend centers in the human β-globin locus [10].

3.2. Fine mapping of the DNA bend site at ERB−2

To obtain insight into the nature of DNA curvature at these sites, we further investigated the sequence features at ERB−2 (Fig. 2A). Three deletion constructs (pERR1–3) derived from the plasmid pPS, which partly (pERR2 and pERR3) or entirely (pERR1) removed the ERB−2 site, were subjected to the circular permutation assay (Fig. 2B). This figure indicates that the bend center of this site is located between the *Hin*dIII site and the right end of the pERR3 insert (position −848) and close to the *Pfl*MI site, although a bending profile remained in the construct where more than half of ERB−2 was removed (pERR2). No bending profile was observed with pERR1. These results suggest that the bend center was indeed located within the ERB−2 site.

The nucleotide sequence of the bend center was examined further with oligonucleotides (Fig. 2C). There were three T/T/T sequences (T1–T3) in the plasmid pPS. Concatenated oligonucleotides containing these T/T/T sequences along with the immediate flanking sequences of T3 in the ERB−2 site were assayed for DNA curvature by polyacrylamide gel electrophoresis (Fig. 2C, left). As summarized in the figure (right), two of the T/T/T sequences (T1 and T3) showed an almost equivalent degree of curvature. The other T/T/T sequence (T2) and two of the flanking sequences of T3 (T3A and T3C) did not show much curvature. In contrast, T3B, a 20-nucleotide sequence located next to T3, showed the highest curvature and the overlapping sequence between T3A and T3B (T3AB) showed moderate curvature, indicating that part of the sequence in T3B is the bend center of this region. There was an A_4 tract in T3B, which may have contributed most to the curvature. Note that there was a half palindromic motif of the ERE (GGTCA) in T3B. The other half (TGACC) was located in the adjacent sequence T3A.

3.3. Nucleosome positions at ERB−2

We previously suggested that DNA bend sites have a potential of phasing nucleosomes [12]. In order to add more evidence to this finding, we used computer software developed for mapping DNA curvature sites and nucleosomes [15,16]. Higher peaks in curvature mapping corresponded generally to the experimentally mapped DNA bend sites (arrowed in Fig. 3, middle), although some did not, the peak between ERB−2 and ERB−1, for example. In the prediction for nucleosome phases (bottom), however, the higher peaks matched well with the experimental sites. All of the predicted nucleosome positions were accompanied by the bend sites.

We further investigated the nucleosomal phases at ERB−2, which were determined by restriction analysis of the ∼146 bp fragments from the nucleosomal core region (Fig. 4). Nucleosomes were prepared from HeLa cells in which the ER gene is

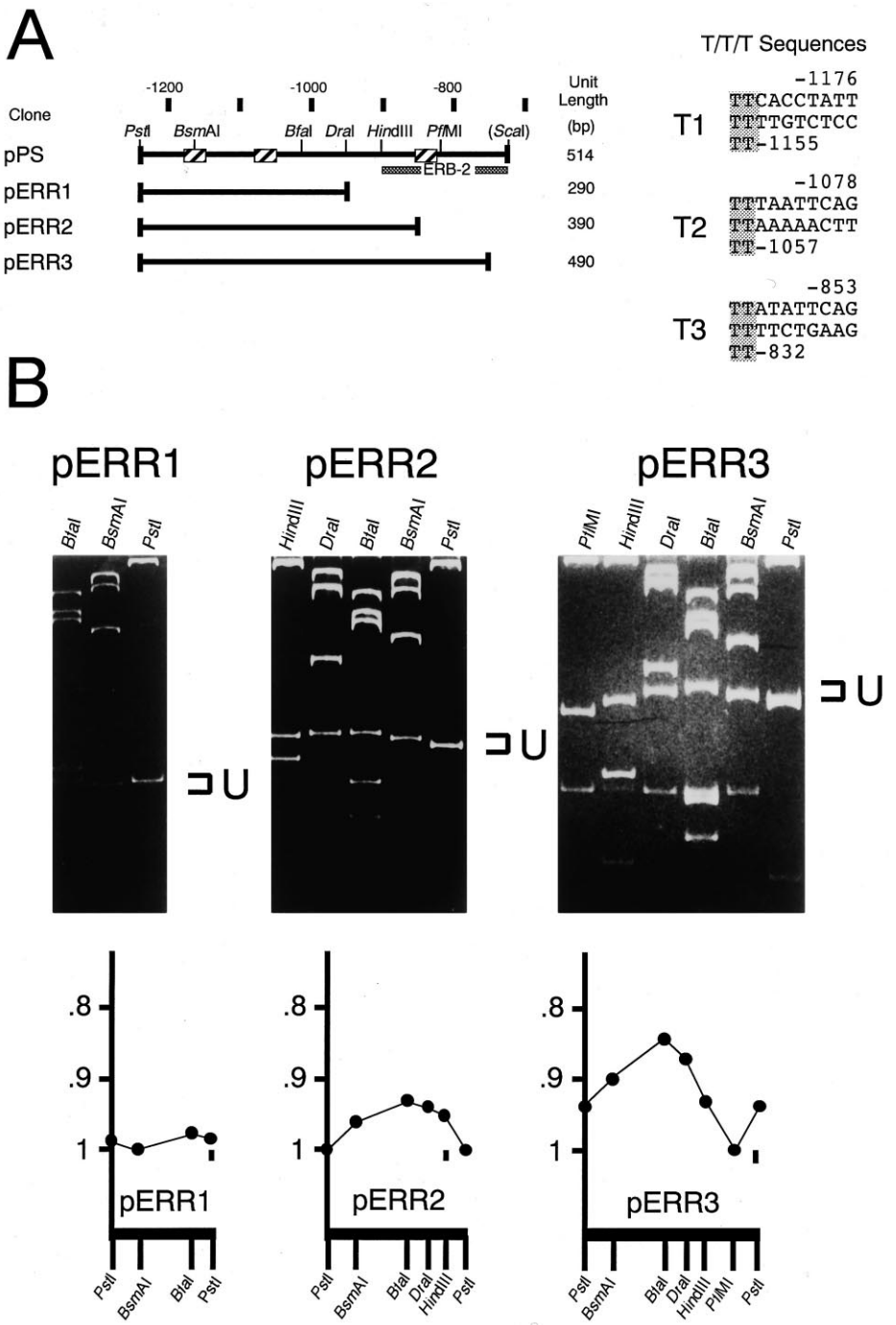

Fig. 2. Sequence features for DNA bending at ERB−2. A: Maps of pPS (nucleotides −1238 to −725 relative to P1) and its deletion constructs pERR1 (−1238 to −948), pERR2 (−1238 to −848) and pERR3 (−1238 to −748). Three T/T/T ($T_2N_8T_2N_8T_2$) sequences in the region (shown as striped boxes) are shown on the right. B: The circular permutation assay with the plasmids pERR1, pERR2 and pERR3. U: unit length fragment. C: Curvature assay with concatenated oligonucleotides (left) and the summary of the assay (right). Twenty-mer duplex oligonucleotides containing the T/T/T sequences −1176 to −1157 (T1), −1078 to −1059 (T2) or −853 to −834 (T3) and the sequences in the flanking regions of the T3 (T3A, T3AB, T3B and T3C) were ligated. The sequence in T3AB was derived from the second half of the T3A sequence and the first half of T3B sequence. A_{20} was annealed with T_{20} and used for the assay and the size standard. The ligation products were electrophoresed through an 8% polyacrylamide gel. The nucleotide sequences of the oligonucleotides used for the curvature assay and the results of the assay are shown below. The TT dinucleotides in T/T/T sequences are highlighted. The ERE motifs are shadowed.

inactive and therefore chromatin in this locus should be packed tightly. The nucleosomes were digested with micrococcal nuclease and the ~146 bp core DNA fragments were recovered and used for restriction analysis. Digestion with *Hin*dIII generated a pair of major fragments in the sizes of 32 bp and 114 bp (Fig. 4A, lane 8). Since none of the restriction enzymes, *Nsi*I, *Dra*I, *Pfl*MI or *Eco*NI (lanes 2, 4, 10 or 12), generated the fragments of ~30–~120 bp long, the position and the direction of the nucleosome of this site was almost uniquely determined (Fig. 4A, bottom). Digestion with *Bst*XI (lane 6) confirmed this alignment. On this alignment, the bend center (shaded box) was overlapping with the

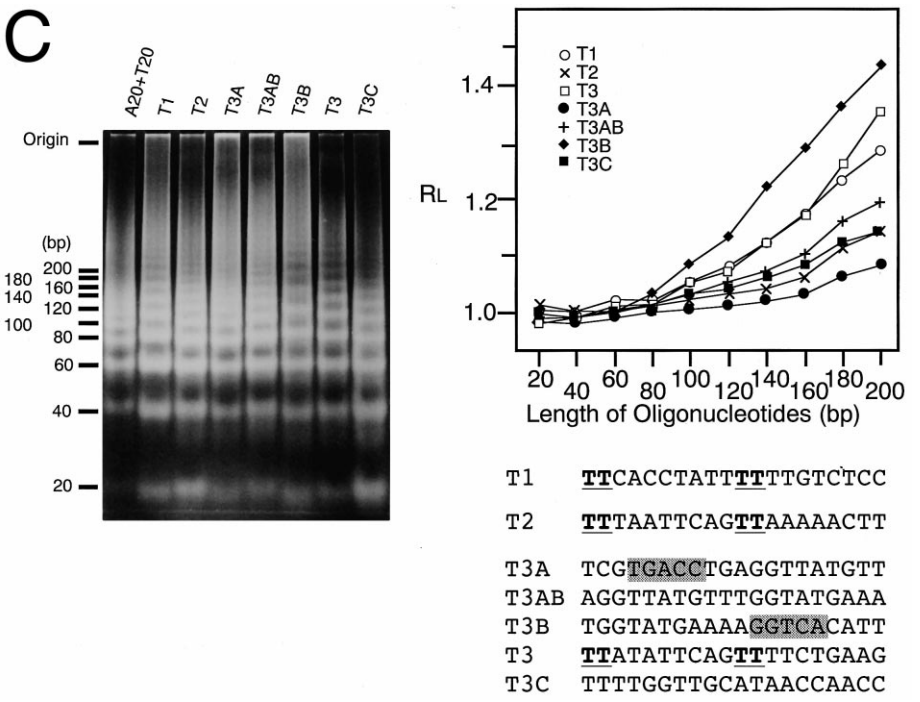

Fig. 2 (*continued*).

core region of the nucleosome (Fig. 4A, top). The patterns by curvature and nucleosome mapping (Fig. 4B, middle and bottom) indicated that the predicted regions matched the experimental bend sites and higher peaks for nucleosomes matched the experimental nucleosome dyad axis (Fig. 4C). The bend center and the nucleosome dyad axis were separated by 10–30 bp (vertical arrows in Fig. 4B and detailed in Fig. 4C).

4. Discussion

The tissue-specific expression of the ER gene is regulated by specific transcription factors which bind to the ERE, located in the promoter region of the ER gene, and other members of the steroid/thyroid/retinoic acid receptor gene families [2]. These sites are arranged as tandem repeats or inverted repeats (palindromes) to make variations of the sites for the transcription factors. The transcription factors which bind to these elements make contact with sites sometimes located hundreds of base pairs apart from each other by forming a dimer [17]. Chromatin structure should play an important role in these interactions as a part of transcriptional regulation. We mapped DNA bend sites in the promoter region of the human ER gene as a first step to understanding the chromatin structure in this region. These sites were shown to be located in a very organized manner: they appeared nearly periodically at intervals of about 680 bp [9–12]. Although the average distance between the bend sites in the ER gene was close to this value, the sites ERB−3 and ERB−2 were shifted towards the P1 promoter, creating a larger distance between ERB−4 and ERB−3 and a smaller than average distance between ERB−2 and ERB−1. This specific distribution of curved regions may have some biological implications, presumably related to dynamic chromatin structure or an open chromatin region which would facilitate interaction of transcription factors with the promoter sequences.

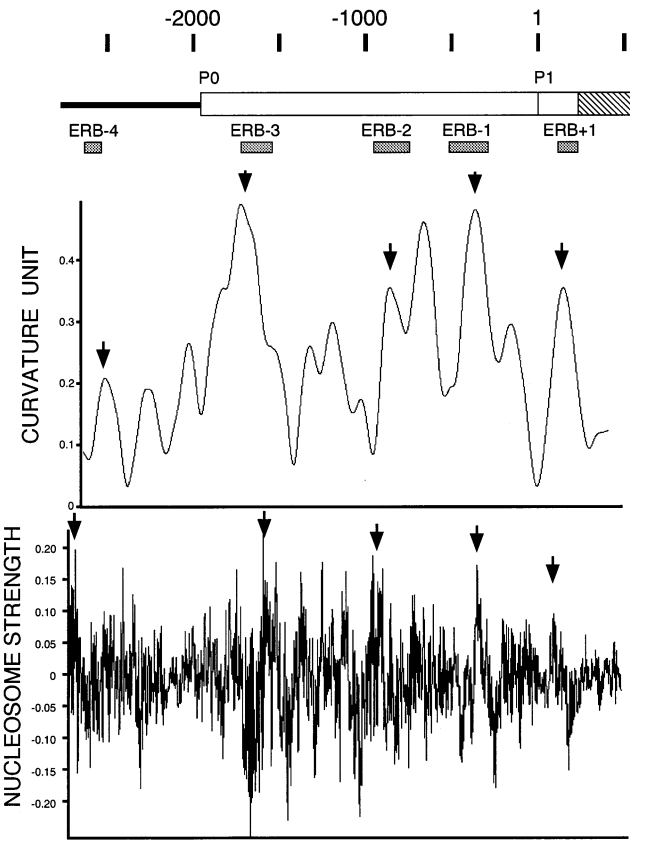

Fig. 3. Prediction of the regions of DNA curvature and nucleosomes. The regions of DNA curvature (middle) and the positions of the nucleosome centers (bottom) were calculated by TRIF 1.00 programs. For curvature mapping, the result of the calculation in a 300 bp scanning window and a 50 bp smoothing window is shown. The higher peaks corresponding to the mapped bend sites are indicated by arrows.

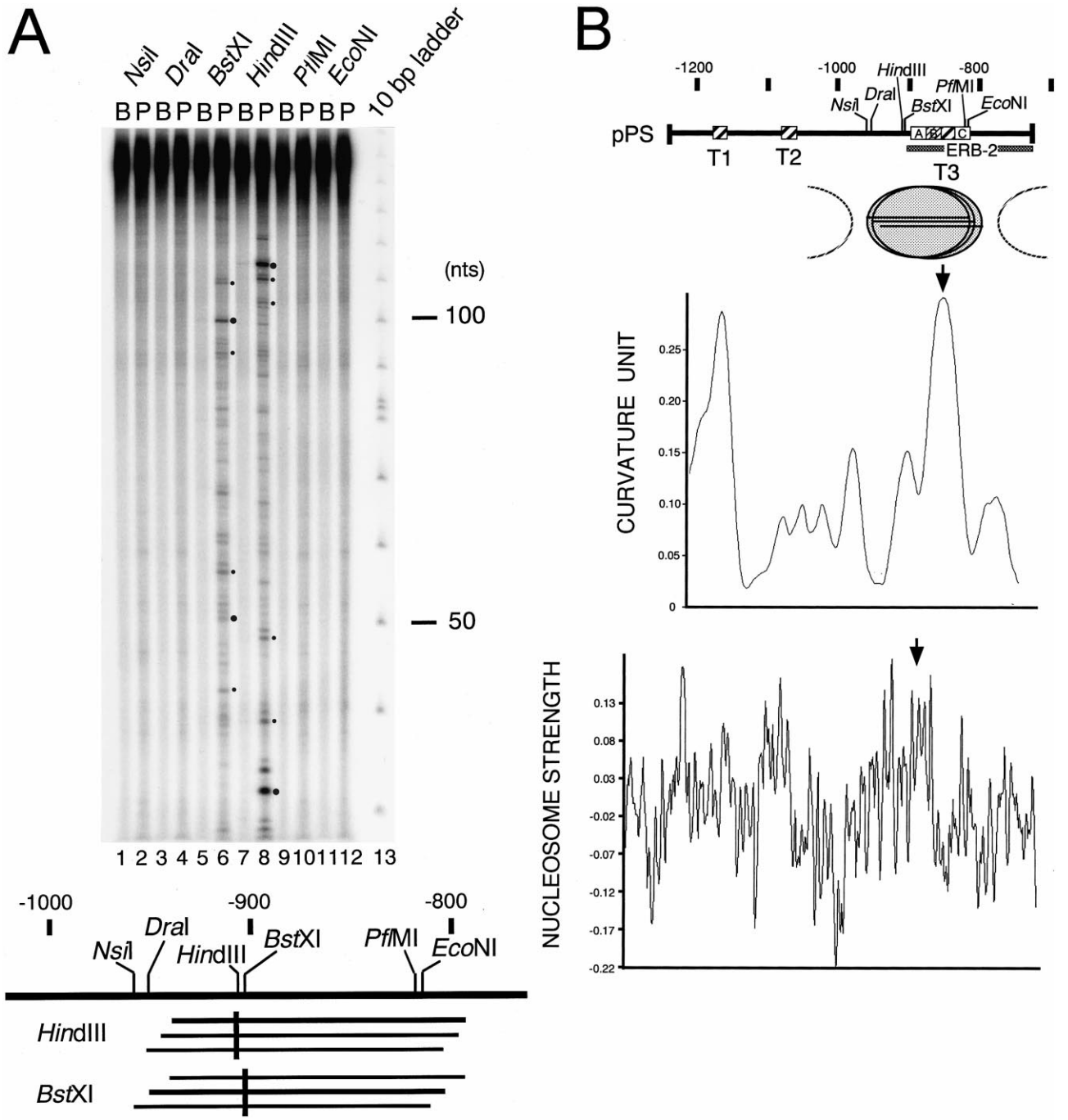

Fig. 4. Nucleosomal positions at ERB−2. A: Mapping of the nucleosome boundaries. The positions were determined for the reconstituted chromatin containing the DNA from the plasmid pPS (P) and the cloning vector (pBluescript, B). DNA fragments from pPS generated by *Hin*dIII or *Bst*XI digestions were 32+114 nt (major), 38+110 nt (minor) and 47+104 nt (minor), or 49+98 nt (major), 38+108 nt (minor) and 56+91 nt (minor) in length, respectively. Major bands are indicated by large dots and minor bands by small dots. The results are summarized at the bottom. B: Summary of the nucleosome positions at ERB−2 (shadowed circle, top) and the results of curvature and nucleosome mapping by TRIF program (middle and bottom). For curvature mapping, a 50 bp scanning window and a 20 bp smoothing window were applied. The regions assayed with oligonucleotides, T3A (shown as A), T3B (shown as B) and T3C (shown as C) are included in the map. C: Enlarged view of B. Complete ERE motifs are shown by solid boxes and an incomplete motif is shown by a hatched box. The $(T)_n$ arrays that contribute to the peaks in the curvature mapping are shadowed.

To analyze the sequence features related to observed DNA curvature we first determined the actual bend center at ERB−2 (Fig. 2). The results showed that the 20 nucleotide sequence directly adjacent to a T/T/T sequence (T3B) is associated with the highest degree of the curvature (Fig. 2C). The sequence contained a short poly(dA) tract, which is most likely to show DNA curvature, and a half of the ERE motif. Another half of the motif is located in T3A (see Fig. 4C), forming a pair of inverted repeats and placing the poly(A) tract in between these motifs. This indicates that DNA curvature might play a crucial role in interactions between DNA elements and protein factors. In this case, DNA curvature

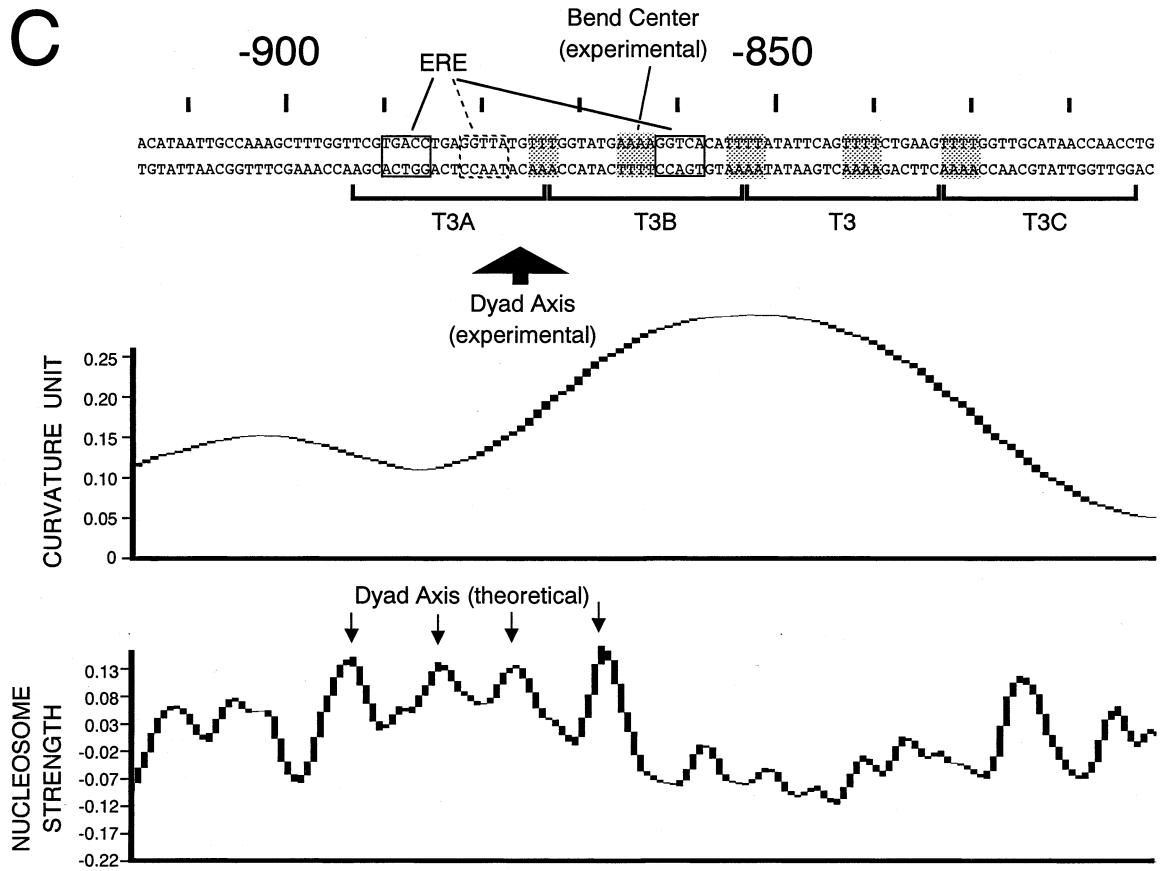

Fig. 4 (*continued*).

should be important for the spatial localization of the factors, e.g. by bringing the factors into proximity with each other for dimer formation. Since the distance between the two half motifs is 23 nucleotides, instead of three nucleotides for the *Xenopus* vitellogenin and many other genes [2], the bound proteins should roughly be located on the same rotational position of double stranded DNA, as in the vitellogenin gene for example, although they are separated by two turns of the double helix. Due to DNA curvature between the motifs, the proteins can presumably form a dimer more readily than without the bend. We estimate the bend angle at the T3B to be approximately 25° from TRIF analysis (data not shown). Since the neighboring sequence T3 also bends, the overall bend angle of this region could be 30° or more. It has been shown that binding of a domain from the *Xenopus* estrogen receptor to the ERE induces a 34° bend [18] and the intrinsic DNA curvature, when arranged properly, can replace the estrogen receptor-induced bending in transcriptional activation [19], suggesting that the intrinsic DNA curvature at the T3B to T3 region in ERB−2 might be important in the function of this region.

Another potential function for the bend sites is localization of nucleosomes. One of the sites in the human β-globin locus was previously shown to determine the nucleosome phase [12]. In the present report, the ERB−2 site was included in the nucleosomal core region and this region and all the other bend sites mapped by gel assays showed a distribution of dinucleotides AA or TT which are typical for nucleosomal core regions and are revealed as higher peaks by TRIF analysis (Fig. 3). Only when the sites were located at the regions of higher probabilities for both DNA curvature and nucleosomal cores, they exhibited the experimentally observed DNA curvature. This adds more evidence that signals for nucleosome positioning are inscribed in the genomic DNA. The curvature regions involved in the nucleosomes do not exactly coincide with them. Rather, about 30 bp separate the respective nucleosome and bend centers. Inspection of the sequences of the bend sites reveals (data not shown) that the sequence sections that belong both to the curved regions and to the nucleosomes have periodically distributed runs of TT or AA dinucleotides in one strand only. Such a sequence arrangement is ideal for both DNA curvature and nucleosome formation [15,16]. The offset of about 30 bp between the bend sites and the nucleosomes means that one of the linkers of the nucleosomes has static curvature. This would be important for three-dimensional organization of the nucleosomes in the chromatin of the promoter region of the ER gene.

Both theoretical and experimental nucleosome dyad axes were located at ERE motifs (Fig. 4C). A pair of half ERE motifs, one complete (GGTCA, solid box) and one incomplete (GGTTA, hatched box), separated by three base pairs creating a palindrome, which is the common feature for other functional ERE motifs [20,21], or a pair of the complete motif separated by 23 bp is located at the region where other proteins have higher accessibility. We found that these motifs can be equally recognized by nuclear proteins from estrogen receptor expressing MCF-7 cells (data not shown). Therefore, placing the factor binding sites at the nucleosome dyad axis

would allow the efficient recognition of the sites while nucleosomes still exist.

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