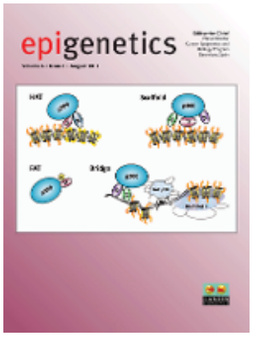

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Highlighting chromosome loops in DNA-picked chromatin (DPC)

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Key words: chromatin, DNA looping, transcription, estrogens, epigenetics, nuclear architecture, lysine demethylation

Growing evidence supports the concept that dynamic intra- and inter-chromosomal links between specific loci contribute to the creation of cell type-specific gene expression profiles. Therefore, analysis of the establishment of peculiar functional correlations between sites, also distant on linear DNA, that govern the transcriptional process appears to be of fundamental relevance. We propose here an experimental approach showing that 17β-estradiol-induced transcription associates to formation of loops between the promoter and termination regions of hormone-responsive genes. This strategy reveals as a tool to be also suitably used, in conjunction with automated techniques, for an extensive analysis of sites shared by multiple genes for induced expression.

Introduction

Most nuclear processes appear to be spatially limited to specific sites that occupy distinct loci within the nuclear space.[1] Remarkably, also the fundamental task of genes, i.e., transcription, is visible as thousands of distinct sites that are currently recognized as sub-nuclear transcription centers, also named "transcription factories."[2] These sites harbor multi-protein complexes that serve numerous genes, with the advantage of concentrating the requested factors to discrete spots and enhance their reciprocal interactions in order to achieve an efficient orchestration of ordered gene expression.[3] In fact, most members of the transcriptional machinery are not evenly dispersed within the nucleoplasm.[4] Transcription complexes dynamically assemble and disassemble, with residence times for the governing factors on target chromatin of only few seconds.[5] It now appears of growing evidence that the transitional compartmentalization of nuclear processes, among which transcription is the prominent albeit not the unique, is one of the basic principles of genome organization in the nucleus.[1]

Among others, chromatin loops represent attractive features for gene regulation because they provide structural supports for the establishment of communications between regions far on linear DNA. In this way, they allow interaction between specific sites on one hand, while they can spatially segregate individual areas from the environment, on the other. Chromosome loops have been implicated in definition of distinct structural compartments referred to as chromosome territories,[6] and relevance of chromatin looping in the regulation of gene transcription has been recently strengthened.[7] Formation of loops has been implicated not only in bringing together far upstream or downstream regions with the gene promoter and body sites, but also in establishing contacts between the 5' and 3' ends of genes,[8,9] in agreement with the now prevalent hypothesis that 3' end-processing factors interact with components of the transcriptional machinery.[10] Moreover, larger chromatin loops are emerging as responsible for separation of specific nuclear regions from each other in order to optimize gene activity.[11]

We propose here an innovative approach to reveal that E_2-induced transcription associates to looping between limited regions of hormone-target genes.

Results and Discussion

Experimental design. Establishment of physical interactions between sites distant on linear DNA has been mainly detected so far by the chromosome conformation capture (3C) technique that utilizes protein/DNA cross-linking of chromatin with formaldehyde, and strategic DNA restriction with suitable enzymes followed by ligation in dilute solutions and PCR amplification of generated fragments.[12] However, this powerful tool requires existence of fitting restriction sites conveniently located along the genes under investigation and close spatial proximity of approached gene loci.

To study how the dynamic positioning of specific genomic sites is related to transcriptional regulation, we have chosen to investigate the response of target genes to 17β-estradiol (E_2) challenge. In fact, it has been recently reported that the promoter

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and termination regions of the breast cancer-associated gene *BRCA1* come close to each other after E_2 addition, an event possibly mediated by formation of loops.[13] Hormone control on gene expression is mainly mediated by its cognate estrogen receptor α (ERα) that, after E_2 binding, contacts cyclically responsive elements on DNA (EREs) [14] and promotes recruitment to chromatin of transcription co-activators that covalently modify the N-terminal tails of nearby nucleosomal histones (histone code).[15] In particular, following ERα targeting to chromatin, a lysine-specific demethylase 1 (LSD1)-dependent DNA oxidation burst is generated with a consequent formation of strand breaks that allow shaping of hormone-sensitive genes.[16]

To investigate changes in the functional correlations between different loci distant on linear DNA, we decided to follow in the present study a different strategy introducing several changes into the protocol initially designed to perform proteomic analysis of protein complexes assembled on specific chromosome sites (PICh).[17] In brief, we cross-linked chromatin from cells treated or not with the hormone according to established procedures,[18] chromatin was then solubilized by sonication to fragments of approximately 500–600 bp and hybridized with a biotinylated oligonucleotide probe followed by capture on magnetic avidin beads. After elution of hybrids, identification of particular DNA sites was realized with PCR. For this reason, we named our experimental approach as detection of loops in DNA-picked chromatin (DPC). In sum, the rationale of our strategy based on the principle that the amount of a specific site in extracted DNA was primarily dependent on its functional proximity to the sequence recognized by the probe. In fact, we reasoned that two sites could be co-captured independently from their location on linear DNA if linked by protein bridges built in the transcriptional output.

In more detail, we hybridized chromatin from 1.0–1.5 x 10^6 cells with 23–30mers selected for their unique sequence in human genome and, at the same time, showing a melting temperature higher than that used in our procedure. In addition, to solve the issue generated by changes in chromatin building possibly induced by hormone challenge, we used more than one probe for any analyzed region observing that the results were independent from the particular probe chosen. We also normalized our results according either to the quantity of DNA extracted from chromatin before hybridization (Input), as well as to the recovery of the hybridizing region when we analyzed rescue of associated fragments.

E_2 challenge induces looping between promoter, enhancer and termination regions of the hormone-responsive *bcl-2* gene. We started our analysis with the already characterized *bcl-2* gene (**Fig. 1A**), where hormone treatment induces bridging between the promoter and the EREs present in the coding region, with looping out of the ~1.5 kb intervening DNA.[16] To assess whether sites different from the promoter and the enhancer were contacted by the estrogen receptor, we preliminarily realized chromatin immunoprecipitation (ChIP) assays with estrogen-deprived $ERα^+$ human breast cancer MCF-7 cells, added or not with 10 nM E_2 for 30 min.[16] As shown in **Supplemental Figure 1**, we found that the *bcl-2* polyadenylation (polyA) region, lacking potential EREs, was specifically enriched in samples from E_2-added cells incubated with anti-ERα antibodies, suggesting that the polyA locus could be associated to the promoter and/or enhancer sites thorough a protein complex containing ERα. Therefore, we hybridized chromatin from quiescent and hormone-challenged cells using biotinylated probes from the promoter or the EREs of that gene, prior to explore the extracted material for presence of different regions. The amount of recovered fragments with the *bcl-2* promoter or ERE sites fell indeed within the expected range (4–8%) for capture of single-copy genes by oligonucleotide probes.[19] Moreover, when hormone was added, the promoter region caught by the probe appeared in a large percentage (roughly 80%) as associated with fragments containing the enhancer sites (**Fig. 1B**). Accordingly, hybridization of E_2-treated chromatin with *bcl-2* ERE probe revealed a comparable association with promoter pieces (**Fig. 1C**). Confirming ChIP results, hormone challenge resulted also into a preferential recovery in both cases of the termination region already evidenced in that experiment (**Fig. 1B and C and Sup. Fig. 2**). We believe that the striking association observed in the experiments shown in **Figure 1B-C** indicates that *bcl-2* promoter, enhancer and polyA sites interact with each other, very presumably because bridged by proteins active in the transcriptional process driven by the hormone.[20] It also appears not unexpected, since local opening of chromatin architecture generally associates to features of biological interest.[21] The role played by chromatin building in chromosome looping was confirmed by the finding that we could amplify exclusively the region containing the probe when we used in the same experiment naked DNA

Figure 1 (See opposite page). Establishment of loops between 5' and 3' regions of the E_2-responsive *bcl-2* gene as revealed by DPC. (A) Graphic representation of *bcl-2* gene where the exons have been evidenced with colored boxes. Arrow (Pr.) indicates the locus where transcription starts. The two estrogen responsive elements (ERE) and the polyadenylation sites (polyA) have been marked under the gene. E_2-triggered changes of the interactions between the promoter, enhancer and termination regions have been drawn on the right. (B) Hybridization of chromatin from quiescent or E_2-challenged (10 nM for 30 min) MCF-7 cells with a probe from *bcl-2* promoter (bcl-2 pr.) to evidence preferential presence of specific gene regions in retrieved DNA. Locations of primer pairs used in PCR reactions (graphically reported on the right) are drawn on top of each gel. bcl-2 intr. symbolizes a model site spread out along the ~250 kb intron. Hybridization of naked (nak.) DNA is also shown. The procedures followed to quantify the amounts of PCR amplified regions represented as % of the associated Input or of the hybridizing fragment are detailed in the Methods Section. (C) Same experiment as in (b), where chromatin was hybridized with a probe from *bcl-2* enhancer region (bcl-2 ERE). Graphics report, as in (B), rescued DNA sites after hybridization with *bcl-2* ERE probe, calculated as % of input, or of the hybridizing region (see Methods). Error bars indicate SEM from at least three independent experiments. Scheme of the 3C experiment realized to assess formation of loops between *bcl-2* promoter, enhancer and polyadenylation sites. Colored boxes representing exons are as in Section (A). Down-pointing arrowheads symbolize *Bgl II* restriction sites. Sites used in shown experiment are reported in colors. Primers for PCRs are depicted with directional black arrows. Length of primer-derived PCR products is detailed on top of each arrow in the middle line. Electrophoretic patterns for the investigated loops and length of the amplified products obtained exclusively in the presence of T4 DNA ligase (lig.) are shown at the bottom as an example representative of three different experiments.

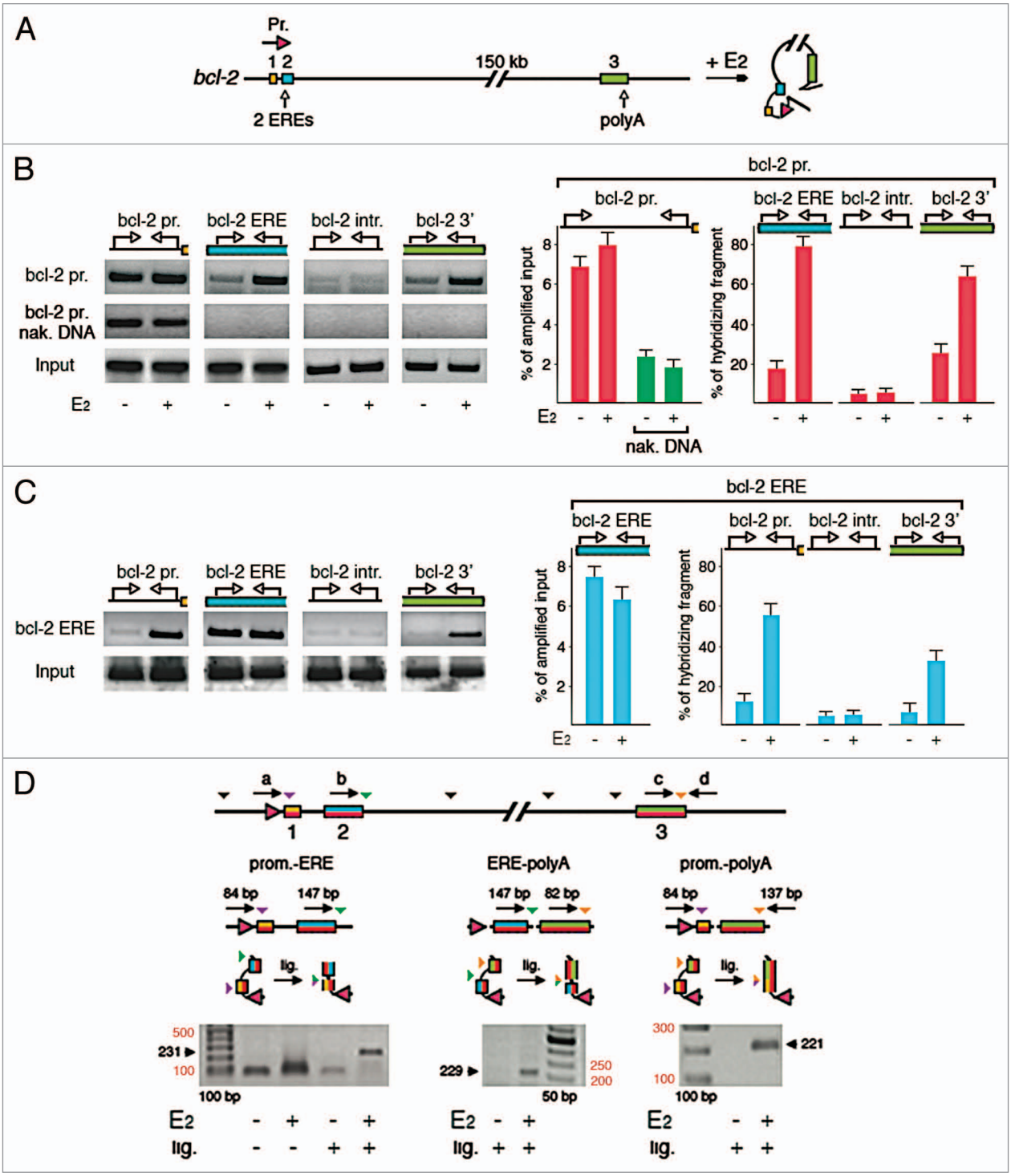

Figure 1. For figure legend, see page 980.

as template (**Fig. 1B**). Moreover, capture efficiency of the complementary site was lower in this case, corroborating the hypothesis that chromatin structure also enhances the interaction between the probe and its complementary locus on DNA. Ultimately, to confirm the establishment of gene loops, we performed experiments using the well-known 3C protocol. The results reported in **Figure 1D** substantiate our interpretation of data obtained by DPC for investigated *bcl-2* regions.

E_2 treatment elicits preferential interaction between hormone-responsive *RIZ* promoter and the polyadenylation sites. To expand the findings described above, we explored the behavior of another already characterized estrogen-responsive gene,

RIZ, that shows two alternative promoters, the more downstream of which (promoter 2 in **Fig. 2A**) is E_2-sensitive.[22] Analysis of *RIZ* gene produced equally interesting results. The captured promoter 2 specifically associated, in fact, with increased amounts (60–70%) of molecules from exons 9a and 10 (where alternative polyA addition occurs) after hormone challenge and, as opposite, a decreased recovery of the E_2-insensitive promoter (promoter 1) could be monitored (**Fig. 2B and Sup. Fig. 3**). In agreement with these data, when we used a probe from exon 9a, we obtained an exclusive increase of promoter 2 DNA after hormone addition (**Fig. 2C**).

Establishment of described loops appeared to be ERα-dependent, since when we realized identical experiments using the ERα$^-$ MDA-231 human breast cancer cells we observed that E_2 addition did not generate a preferential association of *RIZ* promoter 2 with any of analyzed regions (**Fig. 2B**). Notably, in experiments with *RIZ* promoter 1 used as probe, both polyadenylation sites and promoter 2 were diminished in DNA fragments purified after E_2 challenge, suggesting an hormone-dependent overall spatial redistribution of the gene (**Fig. 2D**). Finally, we sustained our data showing that the promoter/enhancer and termination regions of the paradigmatic E_2 target gene *pS2* make similar contacts after hormone treatment (**Sup. Fig. 4**).

E_2 addition affects functional and/or spatial correlations between responsive genes located on different chromosomes. It has been recently shown that liganded-ERα initiates formation of gene networks playing a functional role in the enhancement of estrogen-dependent trancription.[23] Indeed, hormone-induced gene expression requires establishment of intra- and inter-chromosomal interactions and association of responsive genes with inter-chromatin granules.[24] Thereafter, we analyzed more at large the spatial connections between the genes under investigation and found that *bcl-2* enhancer and polyadenylation sites were specifically increased in DNA from E_2-added cells after hybridization with *RIZ* promoter 2 (**Fig. 3A**), while the same sites were captured according to an opposite scheme by *RIZ* promoter 1 probe (**Fig. 3B**). These data are supported by the notion that chromosomes 18 and 1, where *bcl-2* and *RIZ* genes reside respectively, occupy nearby territories[25] that might be presumably involved in the building of hormone-dependent transcription factories.[26] As opposite, the DNA purified from quiescent and hormone-challenged cells revealed irrelevant levels of fragments containing the promoter of the E_2-insensitive *cyp26a* gene (**Fig. 3C**), that lies on chromosome 10 and, consequently, far from chromosomes 1 and 18.[25]

E_2-induced DNA looping of responsive genes is dependent on demethylation of lysine 9 in H3 histone. We have already demonstrated that prevention of demethylation of H3 dimethyl-lysine 9 (H3K9me2) at *bcl-2* promoter and enhancer sites by LSD1 abolishes either the oxidation-driven looping between these sites, as well as hormone-responsive transcription.[16] In order to correlate chromosome looping with the transcriptional output, we initially confirmed the hormone-dependent assembly of LSD1 and the base excision repair (BER) enzyme 8-oxo-guanine-DNA glycosylase 1 (OGG1) on *RIZ* promoter 2 (**Fig. 4A**).[16] Subsequently, we inhibited the LSD1-dependent demethhylation of H3K9me2 with pargyline that, even though not strictly specific to the demethylase, has been successfully used to this aim in previous studies.[27] Pargyline addition abolished, in fact, promoter accumulation of OGG1 and active RNA polymerase II (**Fig. 4B**), as well as its hormone-dependent increased capture of polyadenylation sites (**Fig. 4C**). Hence, at least for E_2-responsive genes, DNA looping between the promoter and termination regions, allowed by demethylation of H3K9me2, seems to play a role in productive transcription.

In conclusion, we are convinced that the described protocol will represent a useful tool for specific and, in association with automated techniques, wide-genome analyses of the transcription-driven repositioning of chromosomes within the nuclear space.

Materials and Methods

Cells. Human breast cancer MCF-7 and MDA-231 cells were routinely grown as already described in reference 16. To evaluate the effect of E_2 challenge, cells were first incubated with phenol red-free DMEM with 5% dextran-charcoal-stripped FCS for 4 days, and then with the same medium containing 0.5% dextran-charcoal stripped FCS for further 6–8 h, prior to be challenged with 10 nM E_2 for 30 min. To inhibit LSD1 activity, 3 mM pargyline was added to the cells for 14–16 h, with or without 10 nM E_2 for 30 min.

Chromatin immunoprecipitation (ChIP). ChIP assays were carried out as previously reported in reference 16. Chromatin from approximately 5 x 10^6 MCF-7 cells was sonicated in order to obtain fragments of 500–600 bp. For each assay, chromatin from a total of 5 x 10^5 cells was used. All bands from ethidium bromide stained 2% agarose gels were analyzed by densitometry and quantified with Total Lab ID software. The list of antibodies used as well as sequences of primers and conditions of PCRs are available upon request. In particular, to accurately measure the amount of immunoprecipitated DNA, PCR amplifications were quantified using an Input calibration procedure: logarithmic serial dilutions of each Input DNA were amplified and plotted against the threshold cycle (formerly established within the range of cycles with exponential duplication of template sequences), thus obtaining a calibration curve. Moreover, in order to minimize saturation of PCR amplifications, AmpliTaq polymerase in ABI buffer (30 mM Tris, 10 mM HEPES, 25 mM KCl, 20 mM potassium L-glutamate, 20 mM ammonium acetate, 1.25 mM DTT, 5% glycerol, 1.25 mM $MgCl_2$, 0.2 mM dNTP) was used in all reactions.[28] The relative amount of any PCR product from immunoprecipitated samples was then calculated as percent of Input by interpolation onto the associated curve described above.

DNA-picked chromatin (DPC). This procedure was performed by introducing the following modifications into the already reported protocol for proteomic analysis of isolated chromatin.[17] MCF-7 cells from semi-confluent 10 cm dishes were cross-linked and collected into 100 mM Tris-HCl (pH 9.4), 10 mM DTT, according to already reported procedures.[16] Prior to sonication, cell pellets were re-suspended into 0.5 volumes of 1x PBS-0.5%

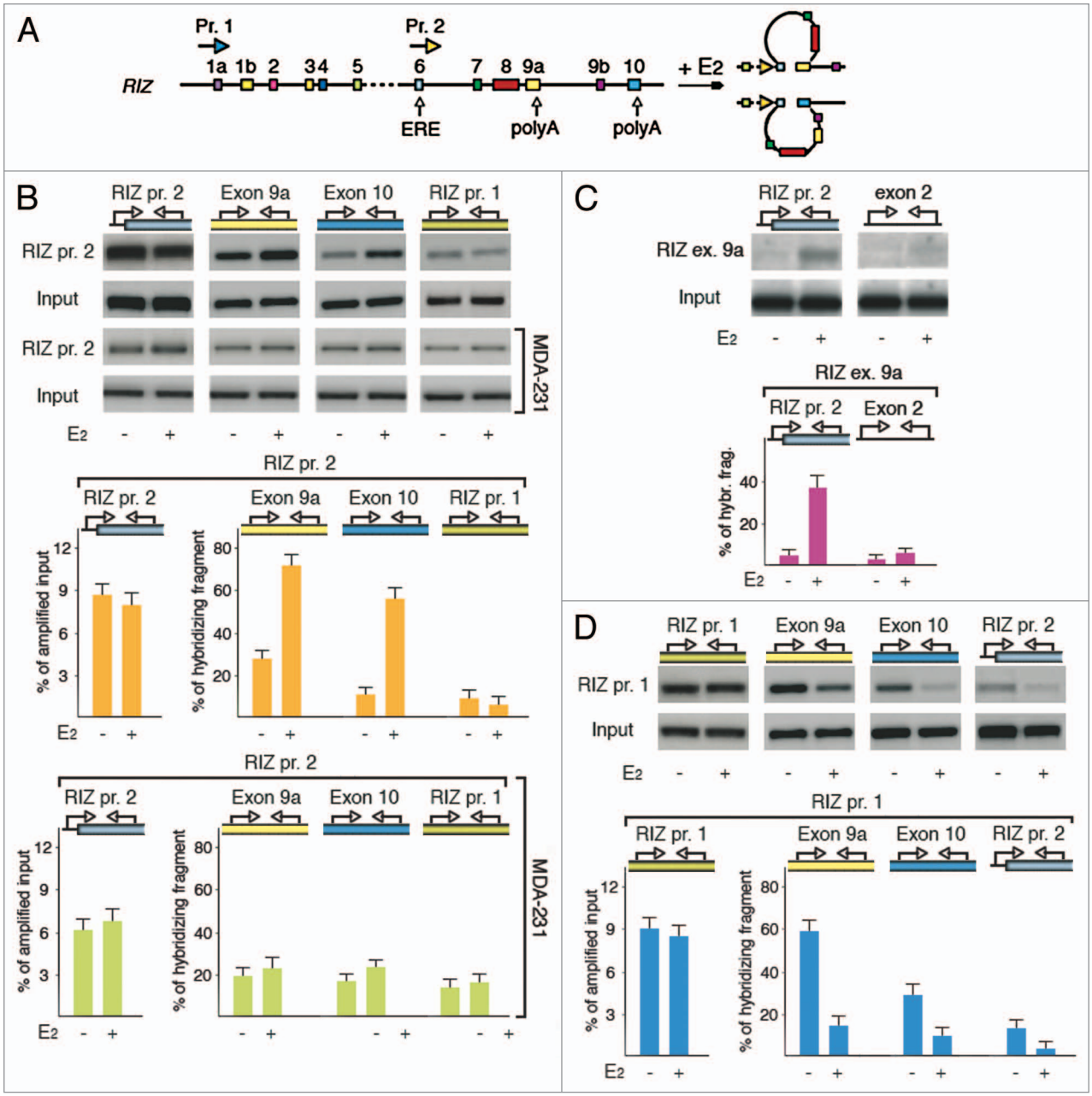

Figure 2. DPC brings to light hormone-dependent looping between the E_2-responsive promoter and the polyadenylation sites of *RIZ* gene. (A) Graphic representation of *RIZ* gene where symbols and E_2-induced conformational changes are as in **Figure 1A**. (B and D) Hybridization of chromatin from MCF-7 or ERα$^-$ MDA-231 cells (where specifically indicated) with a probe from the E_2-responsive (RIZ pr. 2) or insensitive (RIZ pr. 1) *RIZ* promoter shown as a representative gel (on top) or graphically (at the bottom). Exon 9a and exon 10 indicate alternative polyadenylation (polyA) sites. Locations of primer pairs used in PCRs are drawn as in **Figure 1B**. (C) DPC analysis showing formation of a loop between *RIZ* promoter 2 and the polyA site in exon 9a by use of a probe from the latter site. Exon 2 represents a negative control. An illustrative gel is shown on top, with graphic analysis from two independent experiments reported at the bottom.

Triton X-100, with 2 μl of RNase A (20 mg/ml). The mixture was then incubated at RT for 1 h with gentle shaking and at 4°C for 12–16 h before being washed six times in 1x PBS and centrifuged. Pellet was finally re-suspended in 300 μl of sonication buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1% SDS, protease inhibitors). After sonication, samples were cleared by centrifugation at 16,000 g at RT for 15 min and surnatants were collected and diluted in buffers with the following final concentration, 0.4% SDS, 0.1% Sarkosyl, 100 mM NaCl, 2 mM EDTA, pH 8.0, 1 mM EGTA, pH 8.0. 100 μl aliquots (corresponding to 1.0–1.5 x 10^6 cells) were added with high molar excess (1 μM final concentration) of biotinylated oligonucleotide probe, and hybridization was conducted as follows and according to the already reported protocol:[17] 25°C for 3 min, 70°C for 6 min, 38°C for 60 min, 60°C for 2 min, 38°C for 60 min, 60°C for 2 min, 38°C for 120 min, 25°C final temperature. Eluates from samples incubated

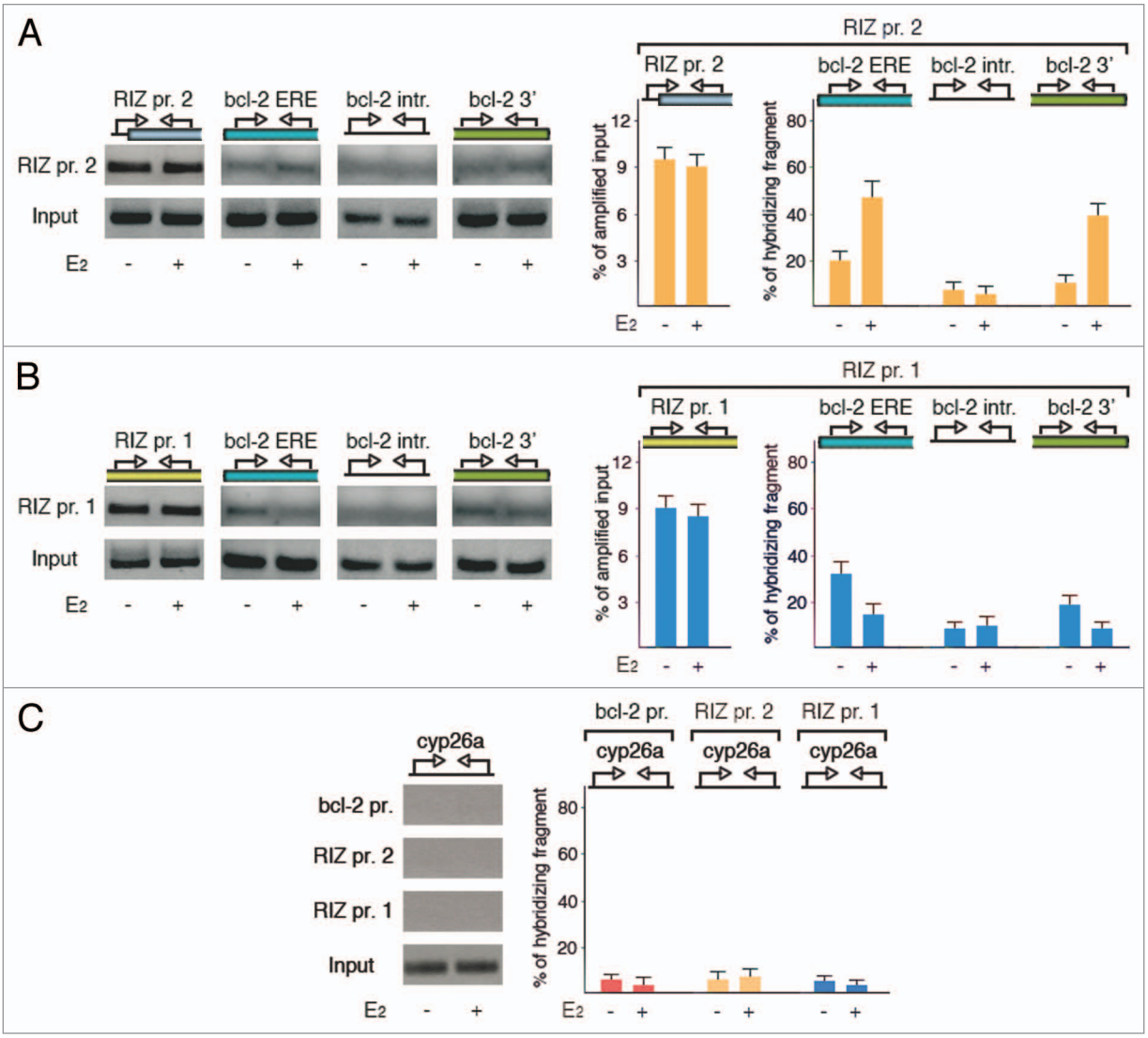

Figure 3. Ability of DPC to highlight inter-chromosomal correlations. Evaluation by DPC of the functional and/or spatial links shows that hormone-responsive genes tend to interact with each other after E_2 challenge.

at RT for 1 h and at 65°C for 10 min were treated with proteinase K and DNA was extracted with phenol-chloroform and precipitated with ethanol, prior to be amplified with PCRs. Quantities of PCR products from hybridized DNA were reported as a percent of amplified Input according to the Input calibration procedure described for ChIP assays. To estimate the association of specific DNA sites with the hybridizing region captured in DPC assays, the proportion of PCR products from any considered region quantified as % of the related amplified Input was compared to the % of Input represented by the hybridizing site amplified by PCR under the same experimental conditions.

The following 5'-biotinylated oligonucleotides were used as probes in shown experiments:

RIZ promoter 1: GGC AGT TAT TGT TGA GAG AAA AGC

RIZ promoter 2: CTC CTG GTC TGG TAC AAT GGG GAA

RIZ exon 9a: GTT GTG CTA TTG CTG CAA ACA CTT AAT

bcl-2 promoter: GAA GCA GAA GTC TGG GAA TCG ATC TGG AAA

bcl-2 ERE: GCC AGG CCG GCG ACG ACT TCT CC

pS2 promoter: CGG GGT CGC CTT TGG AGC GAG AGA GG.

Chromosome conformation capture (3C). Quiescent and E_2-challenged MCF-7 cells (~2.5 x 10^6) were cross-linked with 1% formaldehyde as already reported in reference 16. Centrifuged cells were then re-suspended in 1 ml lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.2% NP-40, protease inhibitors). Nuclei were centrifuged, washed and re-suspended in a 100 µl final volume of *Bgl II* restriction buffer, in the presence of 0.1% SDS, and incubated 15 min at 37°C. SDS was then sequestered with 1% Triton X-100 and digestions with *Bgl II* (100 U) were performed at 37°C for 16–18 h. The enzyme was

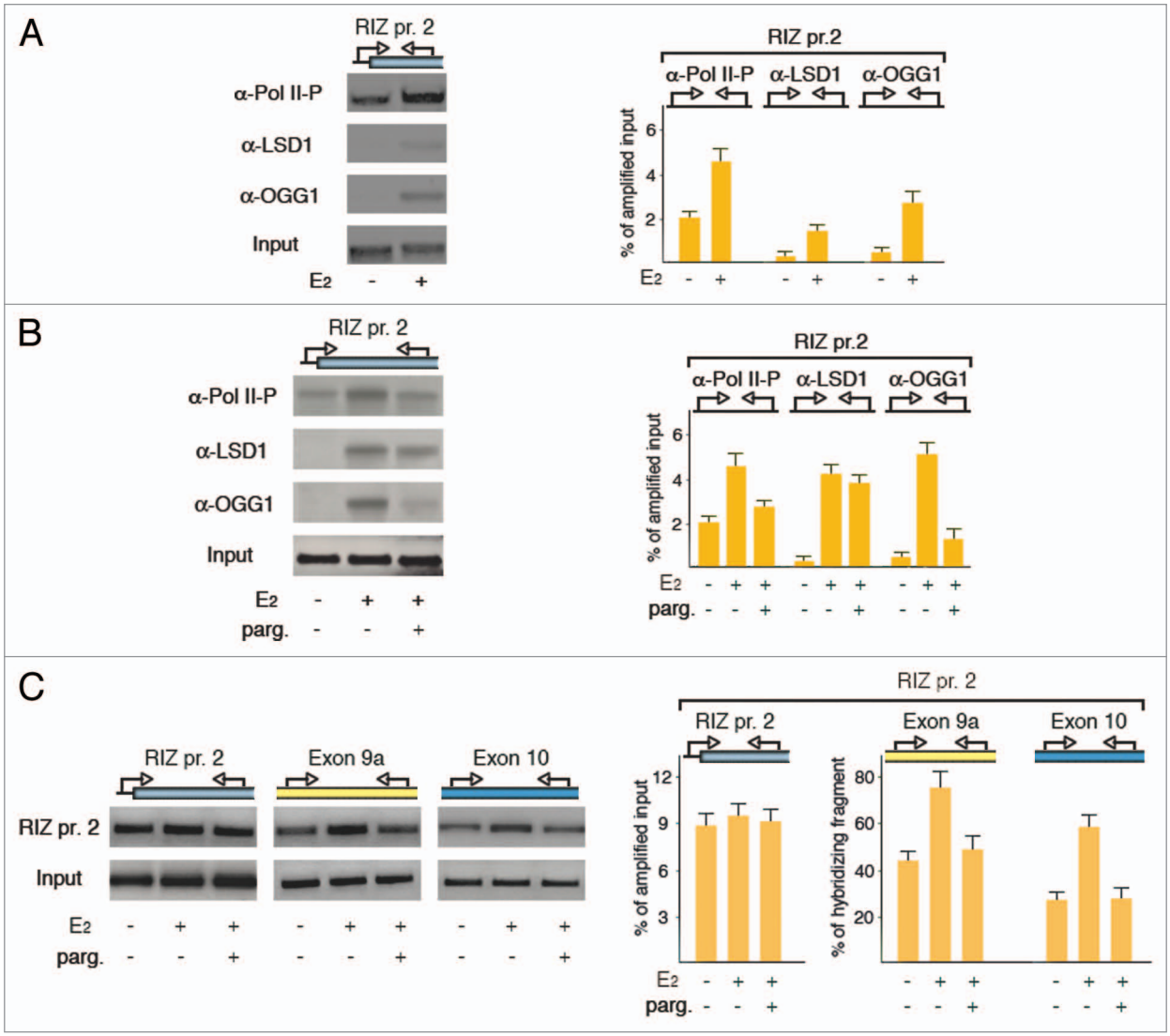

Figure 4. E_2-dependent gene looping is triggered by H3K9me2 demethylation. (A and B) ChIP assays realized with antibodies to the Ser^5-phosphorylated RNA polymerase II (α-Pol II-P), LSD1 and the BER enzyme OGG1 incubated with chromatin from MCF-7 cells treated or not with 10 nM E_2 for 30 min and with or without addition of 3 mM pargyline for 14–16 h. One from two experiments that gave almost identical results is shown in each section. (C) Hybridization of chromatin to observe the effect of pargyline treatment on capture of *RIZ* termination regions by promoter 2 probe.

then inactivated by incubation at 65°C for 30 min with 2% SDS followed by sequestering with 1% Triton-X100. Ligation was realized in diluted volumes (1 ml) in order to hamper intermolecular interactions, using T4 DNA ligase (40 U) at 16°C for 36–48 h. Reactions were stopped with 10 mM EDTA, pH 8.0 and 3U of proteinase K were then added for 5 h at 55°C. Cross-linking was reversed by incubation at 65°C for 12–14 h. DNA was extracted with phenol-chloroform and precipitated with ethanol, prior to be amplified with PCRs and electrophoresed on 1.5% agarose gels. Sequences of primers and PCR conditions are available upon request.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental materials can be found at:
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