Chromatin looping links gene expression to the assembly of transcription factories (Review)

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Abstract. Genes are not randomly dispersed within the nuclear space, instead they occupy precise sites either with respect to the nuclear lamina as well as to each other. This observation stands at the basis of the today well accepted concept of nuclear territories where any chromosome shows reproducible spatial connections with a selection of others in a general picture that meets a functional criterion where genes that answer the same stimuli are grouped in the same sites. In fact, transcription is not visible widely dispersed throughout the nucleus but is gathered in several 'granules', called transcription factories that accommodates ~10 genes concurrently transcribed. This dynamic behavior of chromosomes is allowed by changes in chromatin plasticity that are governed by several classes of proteins that either modify its building or induce post-translational modifications in the protein component of nucleosomes, triggering formation of chromosome loops that modify the location of specific sites along the DNA strand. For example, transcription associated to nuclear receptors benefits of the generation of nuclear ROS that induce nicks following activation of the DNA repair apparatus that enhance helix unfolding and chromosome bridging. In the present review, the role that protocols facing elucidation of chromosome architecture are playing and will play in the near future were highlighted in order to investigate composition of the transcription factories assembled in response of a specific trigger: The estrogen-sensitive transcription was cited but the authors are convinced that the same portrait will be observed with a multitude of (if not all) other stimuli.

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Contents

1. Introduction

The nucleus of human cells harbors 46 chromosomes that are organized into smaller domains, which enable packaging of different structural subunits devoted to different functions. Chromosomes occupy specific locations within the nuclear space, termed chromosome territories, which are further subdivided into chromosomal compartments [topologically associated domains (TADs)] that exhibit specific associations between promoter and enhancer sites (1). It is now universally accepted that location of co-regulated genes in different chromosomes is not casual, as they occupy the same regions within the nuclear space in different cells (2): This painting lies at the base of the notion of 'chromosome territories' where the spatial correlation between chromosomes changes throughout cell life (3). In this model, any gene exhibits different neighbors depending on the specific phase of the cell cycle and, in particular, on its activation state, indicating the presence of a specific molecular apparatus that drives chromatin mobility (4). As a corollary of chromosome territories, it has been also demonstrated that gene expression is grouped in several sites, called 'transcription factories', where genes that share the activating stimuli are concurrently transcribed (Fig. 1) (5,6). Physically, transcription factories can be viewed as nuclear granules with a diameter of roughly 50-100 nm reaching a number that varies between the different cell types and ranges from few hundreds to ~30,000 (7,8). They behave as chromatin hubs where at least two different active RNA polymerases synthesize RNA on two different targets (9,10), and harbor multi-protein complexes that reach local concentrations sufficient to complete ordered expression of co-regulated genes (11). In particular, it has been calculated that any factory contains 8-10 active genes on average (12,13).

2. Chromatin looping and distribution of genes in different territories and transcription factories

The molecular mechanism supporting co-localization of genes within the same factory needs to be investigated in deeper detail; however, it is essentially based on the achievement of a specific flexibility by chromatin that allows creation of several loops able to change location of genes with respect to each other along a chromatin fiber. On this regard, it could be hypothesized that large-scale random movements of chromatin may allow co-activated genes to meet the factories, triggering the multistep process that drives their ordered recruitment to the factory (14,15). This process implies the involvement of different classes of proteins such as chromatin modifiers, transcription factors and, notably, looping factors. Gene looping calls for particular consideration as it governs the precise apposition of enhancers and target genes mainly through different mechanisms where architectural DNA binding proteins contribute to looping by interacting with general or cell-specific factors (16,17). Moreover, occasionally the process involves the direct activity of general transcription factors including the mediator complex and cohesin, that help the looping between enhancers and promoters (18), whereas some other transcription factors, such as ZNF143 and YY1 have been evidenced to enhance chromatin loop formation in order to drive localization of specific loci within the nucleus (19,20). In addition, enhancer and non-coding RNA molecules have been deeply involved in looping between enhancer and promoter sites of specific genes, bringing these two elements into physical proximity in order to allow correct transcriptional processing and indicating chromatin looping as one of the features subjected to enhancer RNA control (21-23). Finally, gene looping also concerns in most cases the 3'-end polyadenylation sites of genes that bridge with promoters and has been revealed to be essential for a correct transcriptional output (24). Interestingly, looping factors make a direct contribution to the recruitment of a specific gene to the factory through the establishment of protein bridges with the transcription factors already located in shared factories (25), governing, in this way, the unequal distribution of transcription throughout the nucleus in order to gain optimized gene expression (Fig. 1) (26).

The separation of nuclear space in chromosome territories is not inelastic and changes in response to environmental stimuli that shape compartmentalization of chromatin in parallel with restriction of the developmental progression (27). In particular, the genome appears widely dispersed within the nucleus in pluripotent stem cells, probably due to the hyper-dynamic nature of looping of chromatin fibers present in these cells (28). However, during lineage specification, a dynamic equilibrium between chromatin mobility and the transcriptional noise underlying specific gene silencing must be reached (29,30). As the specialization process progresses, chromatin compaction increases with large zones of the nucleus devoid of DNA that accumulates along the nuclear envelope and near the nucleolus: An image that correlates with the majority of genes transcriptionally repressed and consequent appearance of several Lamina-Associated-Domains where inhibitory markers prevail (31). These states of chromatin folding are acquired during cell type specialization and are usually reversible depending upon the environmental signal: In this regard an original example that underlines the role played by a specific cell function, is represented by the rod photoreceptor cells where compaction of chromosomes can be observed at the center of the nuclear space instead of periphery. In fact, in these cells, chromatin functions as a physical barrier to the scattering of light when passing through, and its nuclear distribution appears to be in accordance with the regulation of gene expression (32). The development of the concept of Transcription Factory and the characterization of some of such factories shed light on how the transcription factors drive the topological genome reorganization in the context of the control of gene expression. The authors' experience has been focused essentially on steroid receptors, in particular estrogen and retinoic acid receptors, but other examples of TFs mediating the assembly of transcription factories include Nanog (33) and Sox2 (34) in differentiation of pluripotent stem cells, Klf1 (35) in erythrocytes differentiation, Pax3 (36) and MyoD (37) during myogenesis, Pax5 (38) in B cell differentiation.

It is also important to clarify that the transcription factories represent by themselves well-defined entities, but more insight into three-dimensional chromatin changes associated with the activation of transcription could arise from the deeper knowledge about nuclear structures such as the membrane-less organelle (MLOs) (39). Evidence exists suggesting that nuclear condensate formation could be involved in the regulation of various aspects of gene expression, as numerous transcription factors, such as the steroid receptors, undergo Liquid-Liquid Phase Separation, the process leading to MLOs formation. Androgen receptor (AR), estrogen receptor and glucocorticoid receptor (GR) condensates were observed in the presence of the Mediator Complex subunit 1. The formation of condensates is subordinated to the interaction of the receptor with specific chromatin regions in the nucleus. The ligand-bound steroid receptors are translocated to the nucleus, where they form transcriptionally active foci. It has been reported that AR and GR foci are endowed with properties of MLOs (40). Thus, it is conceivable that formation of MLOs including steroid receptor-chromatin complexes contributes to stabilization of the transcription factories architecture and an integrated analysis of both processes could provide comprehensive insight into the regulation of gene expression.

On one extreme side of cellular differentiation and growth stands cancer, where altered nuclear architecture shows an irregular occupancy of the nucleus by chromosomes with altered compaction of chromatin and its DNA content. In particular, cancer cells display changes in the appearance and number of the nucleoli, the first described transcription factories assembled in proximity of the nucleolar organizing regions (41). In fact, an increase in the number of nucleoli parallels a decrease in the stability of contacts between chromosomes carrying rRNA loci, and consequent changes in nuclear organization that may impact adaptive responses. In this regard, cells have evolved a complex machinery to preserve the organization of nuclear space in which chromatin mobility is balanced by factors that restrain its amplitude; however, in transformed cells alterations in bridging between regulatory elements such as enhancers or insulators with promoters are well documented (42). An increase in mobility of cancer genome may also be presumably responsible for

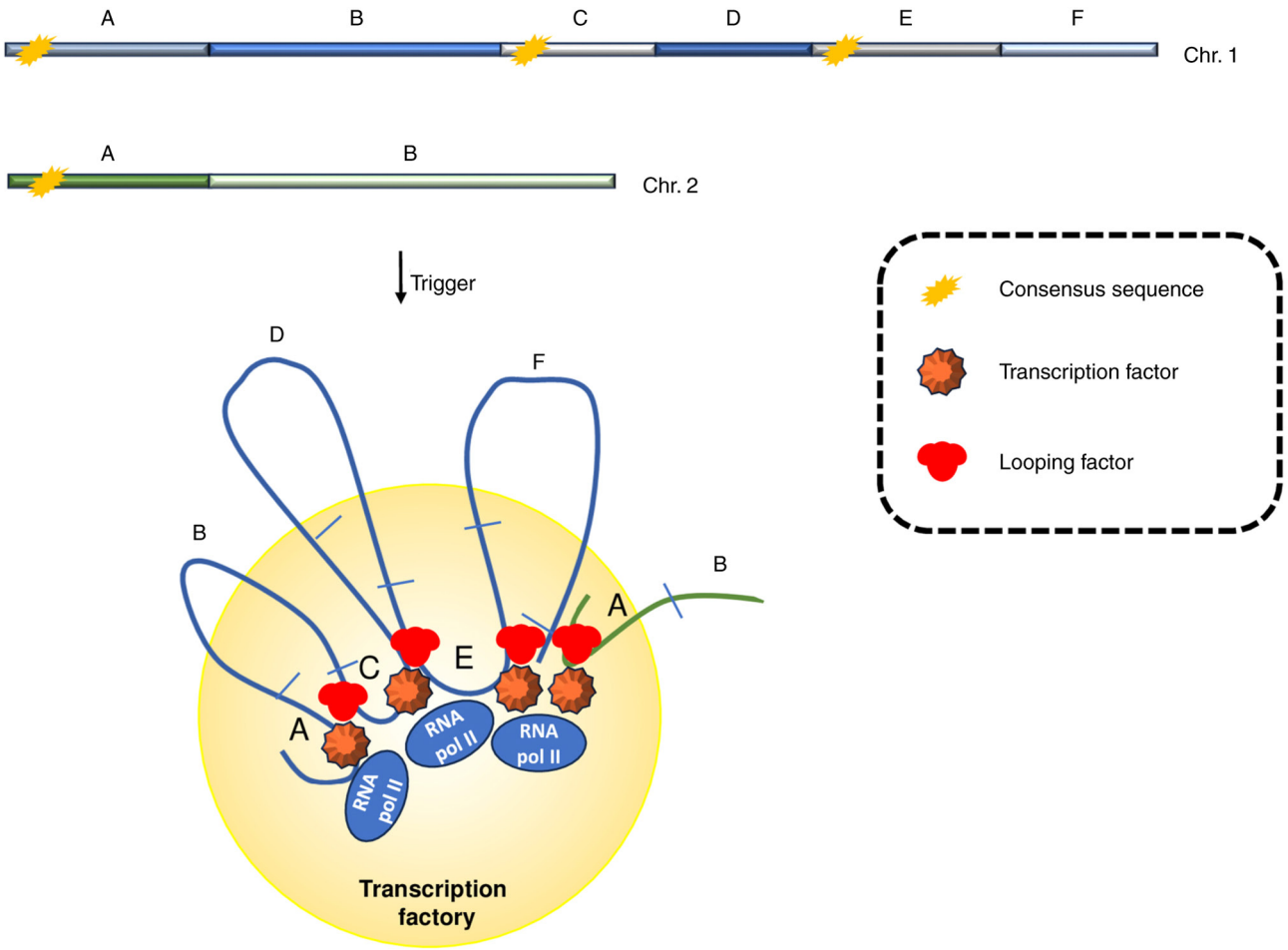

Figure 1. Transcription factory. Graphical representation of a factory where genes from two chromosomes are assembled after a stimulus. Transcriptionally induced genes form the pistils while interleaving ones are the petals of the imaginary flower. The role of chromatin looping factors that interact with the transcription factors located within the factory and ensure the placement of the interacting genes in the multiple transcription complex is evidenced. The shown organization allows an improved efficiency of the entire process, allowing it to be completed by a limited number of active RNA polymerases located in a restricted space, while confirming the view that during transcription the DNA filament slides along a fixed transcriptional complex.

the increased gene expression with consequent phenotypic variability among cancer cells, functioning as the base of tumor progression due to altered contacts with the transcription factors that normally govern gene expression (43). As an example, translocations of *Myc* and *Igh* genes, most frequently found in plasmacytoma cells (44), share the same factories presumably because they require similar transcription factors that start the process of translocation (45,46). The observed increase in genome mobility may be due, at least in some cases, to changes in the activity of genome organizer proteins such as SATB1 that has been involved in the emergence of aggressive tumor phenotypes (47). Finally, a spatial relation between the three-dimensional architecture of the genome and chromosomal alterations has been reported in cancer cells where proximity of regions transcribed at the same time may be used to predict variations in gene-copy number observed frequently in malignant cells (48,49).

3. Experimental strategies to highlight transcriptional looping

Chromosome conformation capture (3C). It is well established that transcription is governed by post-translational modifications at the N-terminal tails of the histone octamer which represents the major protein component of nucleosomes, the fundamental subunit of chromatin. Such modifications follow a precise code (50), and are especially aimed at governing the interaction of transcription factors with the DNA located at the promoter regions of genes to be activated and/or repressed (51,52). Among these modifications, methylation of lysine 4 (K4) and demethylation of lysine 9 (K9) in histone H3, the most protruding tail from the octamer histone disc, are mostly involved in gene activation (53,54). In particular, demethylation of H3K9 is catalyzed by specific demethylases recruited to the regulatory sites of activated genes (55), and produces nuclear reactive oxygen species (ROS) that induce single-strand DNA breaks that rule chromatin plasticity, easing the productive transcriptional output (56,57).

In order to analyze the involvement of gene folding in the expression of responsive genes induced by estrogens, the previously described strategy 'Capturing chromosome conformation', also known as '3C' was first followed (58). In synthesis, the experimental design is based on the quantification of contact frequencies at any time between two loci distant on linear DNA, as revealed by quantitative polymerase chain reaction (qPCR) amplifications yielding incomparably

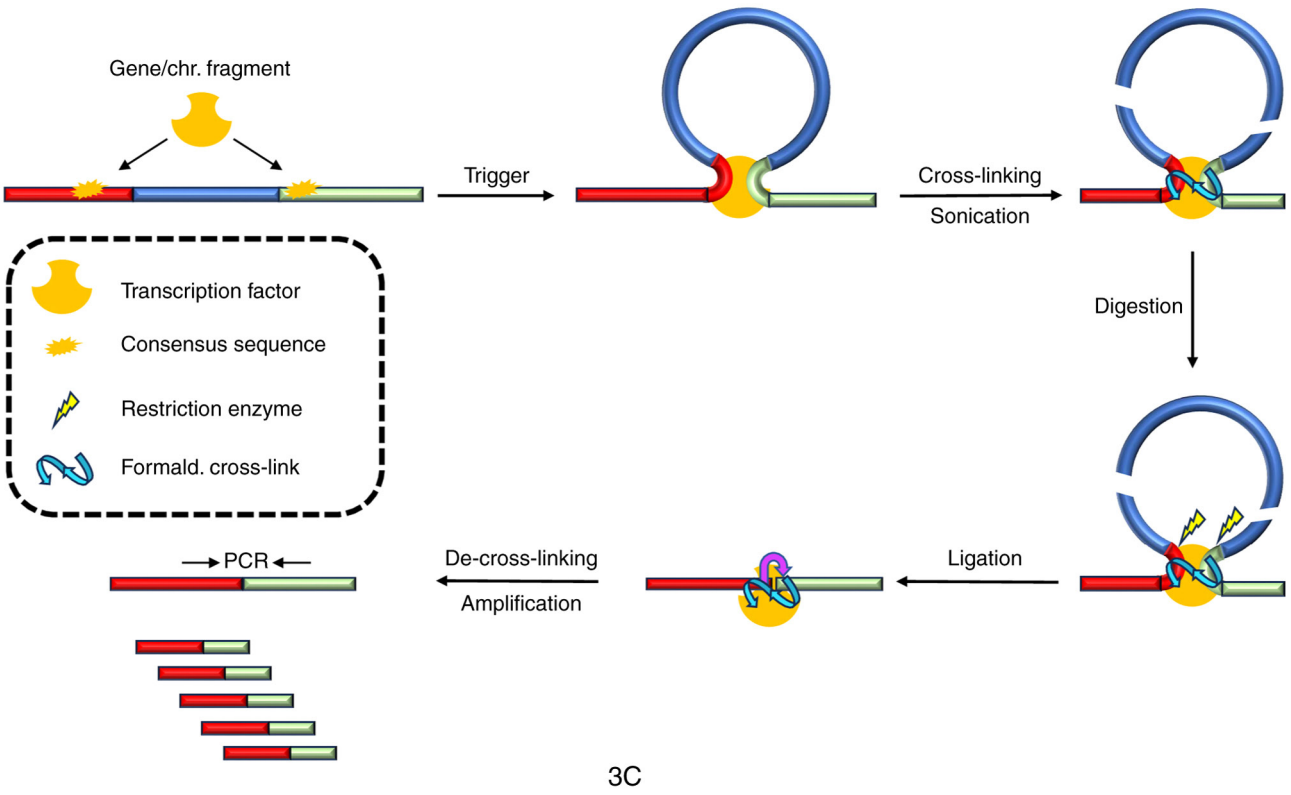

Figure 2. 3C. 3C has been the first experimental technique able to solve with high resolution and reproducibility the challenges derived by the elucidation of the three-dimensional architecture of chromosomes. It is based on the opportunity given by the discovery of dynamic spatial proximity of two sites that, even though are distant on linear DNA, are bridged by transcription factors that target responsive sequences located at strategic sites on DNA. In fact, the chances that sites may be ligated after a restriction treatment increase the closer they are. By comparison with un-induced chromatin, formation of chromosome bridges may be deduced. 3C, chromosome conformation capture.

richer data with respect to previous strategies based on light microscopy, and providing deeper insight into the spatial organization of specific loci (Fig. 2).

The main steps of 3C experimental design may be summarized as follows: i) cross-linking of chromatin using a soluble fixative agent such as formaldehyde to generate covalent bonds between the sites on DNA bridged by proteins; ii) isolation and digestion of chromatin with selected restriction enzymes; iii) ligation of sticky ends of digested fragments at low DNA concentration to favor intramolecular over intermolecular ligations; iv) reverse of cross-linking to obtain purified DNA in order to interrogate the rearranged fragments by qPCR using locus-specific primers encompassing fragments of the sites supposed to be bridged under the investigated experimental conditions; and v) comparison of the PCR results from cross-linked templates with control templates without bridging, to discriminate non-specific ligations. In principle, the cross-linking frequency between two sites on DNA is inversely proportional to their relative distance unless they are put in spatial contact by a particular protein factor. Therefore, a picture of the three-dimensional (3D) architecture of a particular locus may be deduced by comparing linking frequencies from cross-linked chromatin to the control (Fig. 2).

Historically, yeast chromosome III has been the first to be analyzed, and its 3D conformation has revealed proximity between the telomeres that conferred the chromosome the form of a sort of ring (58). Next, 3C experimental approach was adapted for analysis of mammary cells and, even though it can be especially used to detect contacts between sites spanning only few hundred kilobases (59), it confirmed the existence of dynamic chromatin loops between promoters/enhancers and their target genes, conferring chromatin a peculiar conformation dependent on the transcriptional status (60-62).

Upon 3C, it was demonstrated that the estrogen responsive element located ~1.5 kb downstream from the transcription start site of the estrogen responsive gene *bcl-2* bridged through a complex containing the estrogen receptor with the transcription complex located onto the promoter of the same gene. Moreover, looping between the promoter at the 5'-end, and the 3'-end where the polyadenylation site is located, has been widely assessed, supporting the concept that 3' end-processing factors bridge with the transcriptional machinery (63,64).

DNA-picked chromatin (DPC). 3C certainly represents a classical, strong tool to identify gene looping; nevertheless, it requires the existence of suitable restriction sites strategically located along the genes under investigation and setting up the right balance between the concentration of DNA and the restriction enzyme to enhance intramolecular ligations. To try to overcome these bottlenecks, a novel experimental strategy conceived by introducing several changes into the method designed to perform proteomic analysis of multiprotein complexes assembled on chromatin (65) was proposed. This strategy, called DPC (DNA-picked chromatin) by the authors, consisted in cross-linking chromatin from cells challenged or not with estrogens, DNA cleavage to 500-600 base-pair

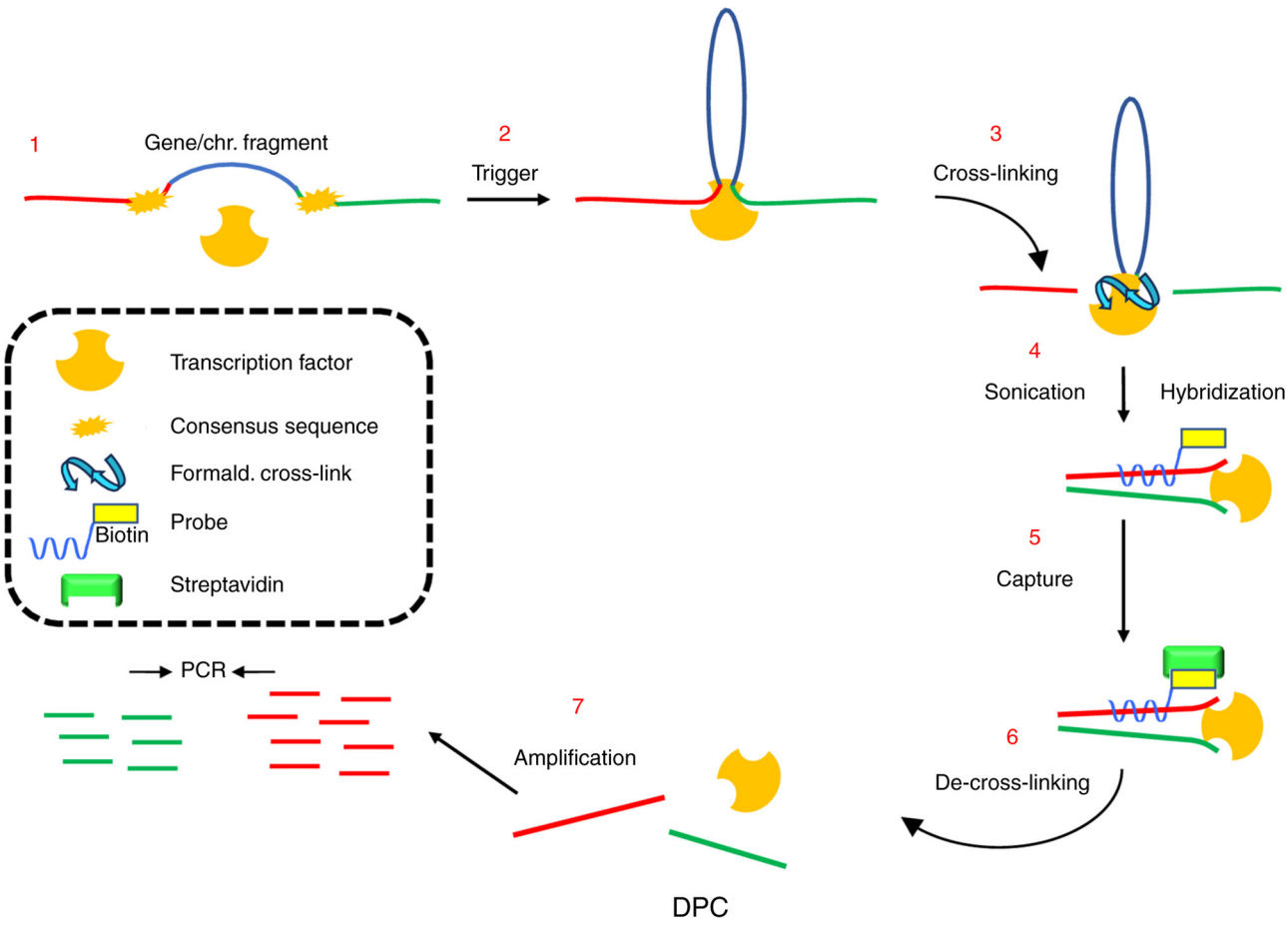

Figure 3. DPC. DPC was conceived as an attempt to try to solve the problems raising from the need of useful restriction sites located in proximity of the analyzed chromosomal loci. It is based on DNA/DNA hybridization to capture with a DNA oligomer used as probe the site under investigation, together with the others that reach its neighborhood after targeting of the transcription factors conveniently cross-linked. As in the chromosome conformation capture protocol, presence of the DNA fragments is revealed by amplification with PCR and comparison of the retrieved concentrations of sites apart from the hybridized one, is considered as evidence of their relative vicinity to the probe site. DPC, DNA-picked chromatin.

fragments by sonication, and hybridization with a biotinylated oligonucleotide probe complementary to the site under investigation, followed by capture on magnetic streptavidin beads (Fig. 3). Presence of DNA loci bordering the site hybridized by the probe was, then, revealed by qPCR amplifications of the DNA extracted and purified from the eluted fragments after de-crosslinking. As a control, PCR reactions were carried out using chromatin before hybridization or naked DNA as template (input), and recovery of the probing region in retrieved DNA from each experimental condition was assumed as the baseline to be compared with the relative amount of retrieved associated sites in the same experimental point. Moreover, to solve troubles emerged by possible changes of spatial chromatin organization throughout transcriptional stimulation, the use of multiple probes was recommended. In summary, the strategy assumes that two sites can be co-captured by the probe only if they are bridged by proteins recruited upon the transcriptional trigger, independently from their distance on linear DNA, and can be imagined as a sort of chromatin immunoprecipitation (ChIP) where the antibody is substituted with the DNA probe (Fig. 3) (66).

Owing to DPC strategy, the spatial relationship between the enhancer and polyadenylation sites of *bcl-2* gene upon estrogen addition was first analyzed and it was observed that the last was increased in the DNA purified after hybridization with the enhancer probe (65). More important, this technique allowed to assess the connection within the nuclear space between this gene, located on chromosome 18, and another estrogen-sensitive gene, *RIZ*, located on chromosome 1, and the authors were able to detect an increase of *bcl-2* enhancer and polyadenylation sites when the rescued DNA hybridized with *RIZ* promoter was used as bait (65). Noteworthy, two different chromosomes, 1 and 18, have been demonstrated to occupy contiguous territories within the nuclear space and previous data by the authors suggested that this is dependent on assembly of the cognate transcription complex after hormone stimulation (67). It was also assessed that the demethylation of H3K9me2 is essential to the formation of transcription-induced looping of hormone-dependent genes (66).

Circular chromosome conformation capture (4C). Having demonstrated the intimate correlation between changes induced on chromatin flexibility by transcription (through induction of single-strand breaks by an increase of ROS production due to the demethylase activity) and establishment of loops that allow allocation of concurrently transcribed genes within the same transcription factories, the further step is represented by the identification of the genes involved in the assembly of such

Table I. Although the 4C appears undoubtedly as the most performing method to discover and analyze new transcription factories, it should be reiterated that 3C and the less widespread DPC techniques still remain very effective tools when a specific target is under investigation.

Technique	Advantages	Limits
3C	Easy to use, high sensitivity, inexpensive	Possible unavailability of restriction sites, undesired structure alteration due to cross-linking, informative only for two known sites
DPC	Independent on availability of restriction sites, free of possible experimental artifacts, very fast, high sensitivity, negligible costs	Informative only for two known sites, limited by the interference of some three-dimensional chromatin structure
4C	Allows a genome-wide analysis of the transcription factories	Possible unavailability of restriction sites, rather expensive, requires preliminary '*in silico*' analysis

4C, circular chromosome conformation capture; DPC, DNA-picked chromatin; 3C, chromosome conformation capture.

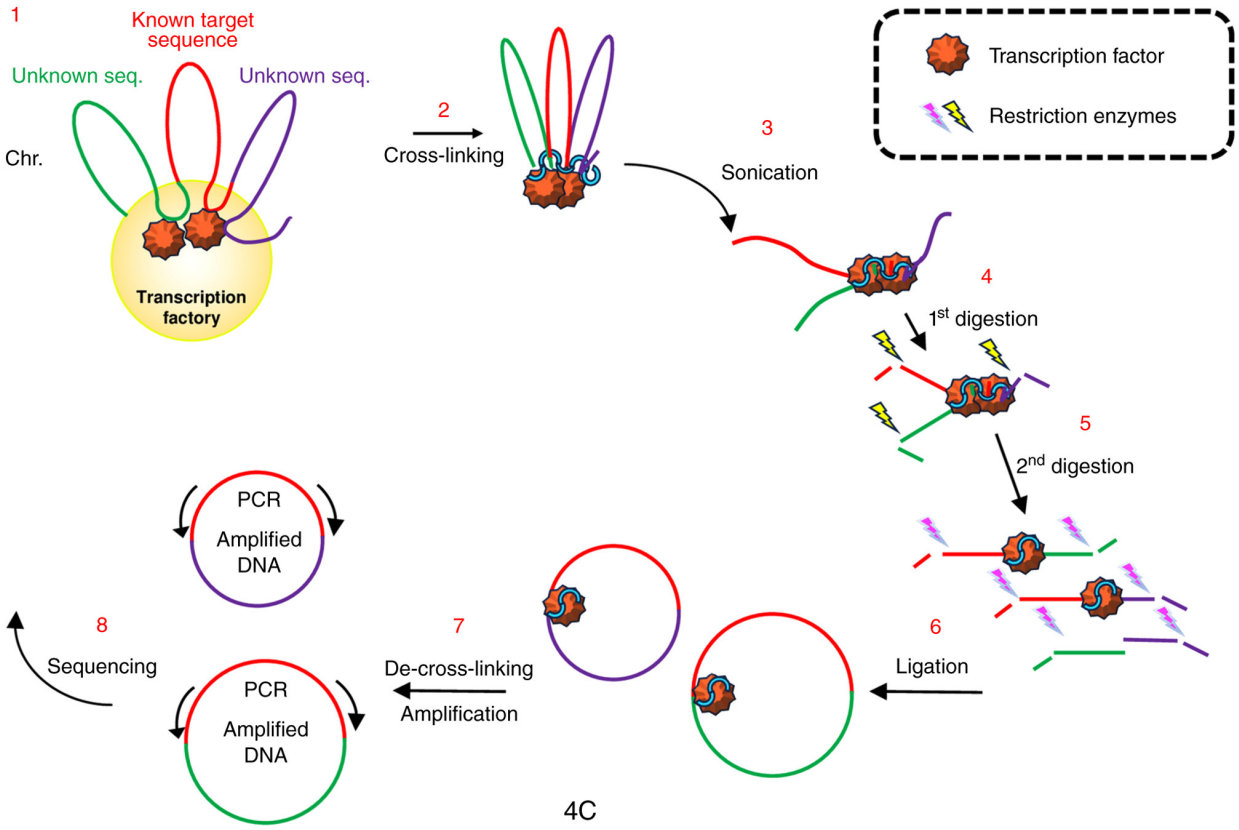

Figure 4. 4C. 4C represents an evolution of the chromosome conformation capture technique and solves the 'one vs. one' point of view changing it in 'one vs. all'. In fact, through the use of a second restriction enzyme that reduces the length of fragments generated by the first restriction treatment, it permits circularization of the DNA fragments bridged by transcription factors after an inducing stimulus and, then, discovery of all the sequences recovered after chromatin de-cross-linking. The circularized fragments are first amplified by PCR with strategic primers the span both ends of the site used as probe and, then, are sequenced to highlight all the genes placed in contact with the bait and, for this reason, ligated. 4C, circular chromosome conformation capture.

factories, starting by a gene whose responsiveness to the investigated trigger is universally recognized (in the present case, the bait is represented by an estrogen-sensitive gene). In fact, as has been graphically represented in Fig. 1, if the estrogen responsive factory is imagined as a flower with the transcribed genes being the pistils and the intervening DNA the petals, then the goal should be the elucidation of the genes that form the factory/flower establishing contacts to the bait (Fig. 3).

To this aim, since either 3C as well as DPC focus on interactions between two already known loci (for this reason called 'one versus one') and can, then, be used exclusively to detect spatial interactions between already known regions (68), an-omics approach is needed, using the products generated by the 3C or DPC procedures as a library that needs further treatment. On this regard, a growing family of related strategies has been reported, among which the 3C-on-chip (69), and the open-ended 3C (70),

mostly based on circularization of the generated chromatin fragments using a second restriction enzyme. Thus, the authors' attention will be now pointed to the 4C experimental approach that represents a paramount protocol to assess the involvement of co-responsive genes within the same transcription factory (a 'one versus all' approach) (71). In synthesis, the procedure may be summarized as follows: Cross-linked chromatin is treated to generate circular DNA molecules from restricted hybrid fragments by use of a second restriction enzyme under high ligase concentration and prolonged incubation times. After reversal of cross-linking, nested PCR primers spanning the opposite ends of the DNA site chosen as probe are used to amplify any sequence fallen in strict proximity with the bait (also called the 'viewpoint') and then, ligated. The amplified ligation products are sequenced to assess all the spatial partners of the gene (or a specific locus of it) under investigation, providing the identity of all the pistils of the flower (Fig. 4) (72).

4. Conclusions and future perspectives

4C has been demonstrated to represent a useful device to highlight either short-range interactions as well as long-range spatial cross-talks between very distant sites (73); therefore, it appears as the strategy of choice to obtain a complete detection of genes that co-localize with the estrogen-responsive gene *PS2* after hormone challenge (74). The same strategy will presumably allow assessment of components of any of the transcription factories established by any transcriptional trigger throughout cellular life-span, either in physiological as well as pathological conditions.

In conclusion, the scope of this brief review was to emphasize the importance of studying the three-dimensional structure of chromatin with the hope of providing a succinct summary, based on the authors' experience of the evolution of the principal methods used to deepen knowledge about this issue; the respective advantages and limits have been highlighted in Table I.

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Availability of data and materials

Data sharing is not applicable to this article, as no data sets were generated or analyzed during the current study.

Authors' contributions

BP conceived, wrote and edited the manuscript. AM discussed and revised the manuscript and prepared figures. GC discussed and revised the manuscript. All authors read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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