Chromatin organization and transcriptional activation of *Hox* genes

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Abstract: Spatially and temporally programmed expression of the *Hox* genes along the antero-posterior (A-P) axis is essential for correct pattern formation during embryonic development. An accumulating body of evidence indicates the pivotal role of spatial chromatin organization for the coordination of gene regulation. Recently, chromosome conformation capture (3C) technique has been developed and opened a new way to study chromosomal interactions in the nucleus. In this study, we describe 3C method we applied in F9 embryonic teratocarcinoma cells and demonstrate that the chromosomal interactions at *Hox* loci are successfully detected. Interestingly, at *Hoxc* loci, the abundance of intrachromosomal interactions with neighboring fragments was drastically decreased when the genes are expressed. These results indicate the possibility of the dynamic pattern of chromosomal interaction in association with the transcriptional regulation of *Hox* genes.

Introduction

Regulation of transcriptional activation in higher eukaryotes involves long-range genomic interactions (Carter *et al.,* 2002; Dekker 2008; Kleinjan *et al.,* 2008). In many cases regulatory elements are located at large genomic distances from target genes, or even on different chromosomes. Several studies have recently confirmed that distant elements function by engaging in direct physical interactions with their target genes (Tolhuis *et al.,* 2002; Lomvardas *et al.,* 2006; Chavanas *et al.,* 2008; Boney-Montoya *et al.,* 2010). The chromosome conformation capture (3C) method has provided an opportunity to map these interaction networks.

3C method uses formaldehyde crosslinking to covalently link interacting chromatin segments in intact cells (Dekker *et al.,* 2002). Crosslinked chromatin is then digested with an appropriate restriction enzyme. The digestion is followed by ligation under diluted DNA concentration, which promotes intra-molecular ligation of cross-linked fragments. Each ligation product reflects an interaction between two genomic loci and can be analyzed and quantified by PCR.

Hox genes are master regulators of development and play pivotal roles during adult tissue differentiation (Wang *et al.,* 2009). In mammals, there are 39 *Hox* genes organized into 4 genomic clusters of 13 paralogue groups. During development, the expression of *Hox* genes is regulated both spatially and temporally in an order correlating with the location of the genes from 3' to 5' in the clusters (Izpisúa-Belmonte *et al.,* 1991; Gaunt *et al.,* 1996). This colinearity strongly suggests that chromatin structure plays an important role in their regulation. For the study of collinear *Hox* gene expression in response to retinoic acid, mouse or human derived embryonic carcinoma (EC) cells has been used (Stornaiuolo *et al.,* 1990; Boncinelli *et al.,* 1991). Differential activation of certain specific *Hox* genes in murine F9 teratocarcinoma stem cells has been reported since 1980s (Breier *et al.,* 1986; Murphy *et al.,*1988). Upon RA treatment, however, comprehensive sequential analysis of whole *Hox* gene expression patterns along the cluster has not been reported yet.

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In this study, we examined RA-induced *Hox* gene expression pattern in F9 cells and employed 3C to analyze spatial organization at *Hox* loci to better understand how chromosome conformation changes upon *Hox* gene activation.

Materials and Methods

Cell culture

Murine F9 teratocarcinoma cells were cultured in Dulbecco's Modified Eagles Medium (WelGENE Inc., Daegu, Korea) supplemented with 10% FBS (Fetal bovine serum; WelGENE Inc., Daegu, Korea) and 100 ug/ml penicillin-streptomycin (WelGENE Inc., Daegu, Korea), at 37°C, 5% CO$_2$ condition. The cells were induced to differentiate by addition of 5×10^{-7} M retinoic acid (RA) to the medium. After the RA treatment, the cells were harvested everyday starting at Day 1 until Day 4. The cells with no treatment were harvested and used as controls.

Total RNA isolation and gene expression analysis using RT-PCR

Total RNAs were isolated from RA-treated or untreated F9 cells using RNA-bee reagent (Tel-Test, Inc., Friendswood, TX, USA). Reverse transcription (RT) was performed with 2 ug of the RNA and cDNA was generated by using the ImProm-ll^{TM} Reverse Transcriptase (Promega, Madison, WI, USA). PCR amplification was performed under the following conditions: initial denaturation for 2 min at 94°C, followed by 30 cycles of 94°C for 40 sec, 58°C for 30 sec and 72°C for 1 min. PCR was repeated with three different set of samples. All PCR primers used for detecting *Hox* gene expression levels were described previously (Yu *et al.,* 2009). For quantification, Multi Gauge V3.0 software (Fuji, Tokyo, Japan) was used.

Chromosome conformation capture (3C)

The 3C assay was performed as described previously (Dekker *et al.,* 2002) with minor modification. In brief, single cells harvested from indicated conditions were crosslinked with formaldehyde and the DNA-protein complex was digested with *Hin*dIII, which was followed by ligation at diluted DNA concentration. For each set of experiment, RA-treated (Day 1 and Day 3) or untreated F9 cells (approximately 1×10^8 cells) were harvested and filtered through a cell-strainer

(40 mM, SPL #93040; SPL lifesciences, Pocheon, Korea) to obtain a homogeneous single cell suspension. Formaldehyde (37%; Biosesang, Seongnam, Korea) was added to 1%, and the samples were crosslinked for 10 min at room temperature. The reaction was quenched by the addition of glycine to 0.125 M. The cells were harvested, washed with PBS and lysed in 5 ml of ice-cold lysis buffer (10 mM Tris-Cl [pH 8.0], 10 mM NaCl, 0.2% NP-40 [pH 8.0]) containing complete protease inhibitors (Roche, Indianapolis, IN, USA). Isolated nuclei were washed with appropriate restriction buffer and resuspended in same buffer (0.5 mL per 1×10^7 cells) containing 0.3% SDS and incubated for 1 hr at 37°C while shaking. Triton X-100 was added to 1.8%, and the nuclei were further incubated for 1 hr at 37°C to sequester the SDS. The crosslinked DNA was digested overnight with the restriction enzyme *Hin*dlll (400 U per 1×10^7 cells; TAKARA, Otsu, Shiga, Japan). An aliquot of DNA was collected before and after the digestion to determine the digestion efficiency. The restriction enzyme was inactivated by the addition of SDS to 1.6% and incubation at 65°C for 20 min. The reaction was diluted with ligase buffer (50 mM Tris-Cl [pH 7.5], 10 mM MgCl$_2$, 10 mM DTT, 1 mM ATP), Triton X-100 was added to 1% and incubated for 1 hr at 37°C. The DNA was ligated using T4 ligase (8,000 cohesive-end units; TAKARA) for 4 hr at 16°C followed by 30 min at room temperature. Proteinase K was added, and samples were incubated overnight at 65°C to reverse the crosslinks. The following day, samples were incubated for 30 min at 37°C with 30 μL of RNase A (10 mg/mL), and the DNA was purified by phenol extraction and ethanol precipitation. The resulting template represents a library of all possible ligation products, which are then detected by PCR. Each experimental set was prepared in triplicate. The DNA concentration was carefully determined using a Nanodrop spectrophotometer. Mouse BAC clones carrying *Hoxa* and *Hoxc* cluter genes (RP23-39E6 and RP24-459N19, respectively, CHORI BACPAC, Oakland, CA, USA) were used as 3C control templates. Two microgams of the BAC DNA was digested with *Hin*dIII and religated so that a collection of all possible ligation products are present in equinolar amounts.

PCR analysis of the 3C templates

To determine the digestion efficiency of each 3C template, an aliquot of undigested and digested samples was decrosslinked, and DNA was isolated using phenol/chloroform extraction followed by ethanol precipitation. For assessing the digestion efficiency, 150 ng of DNA was
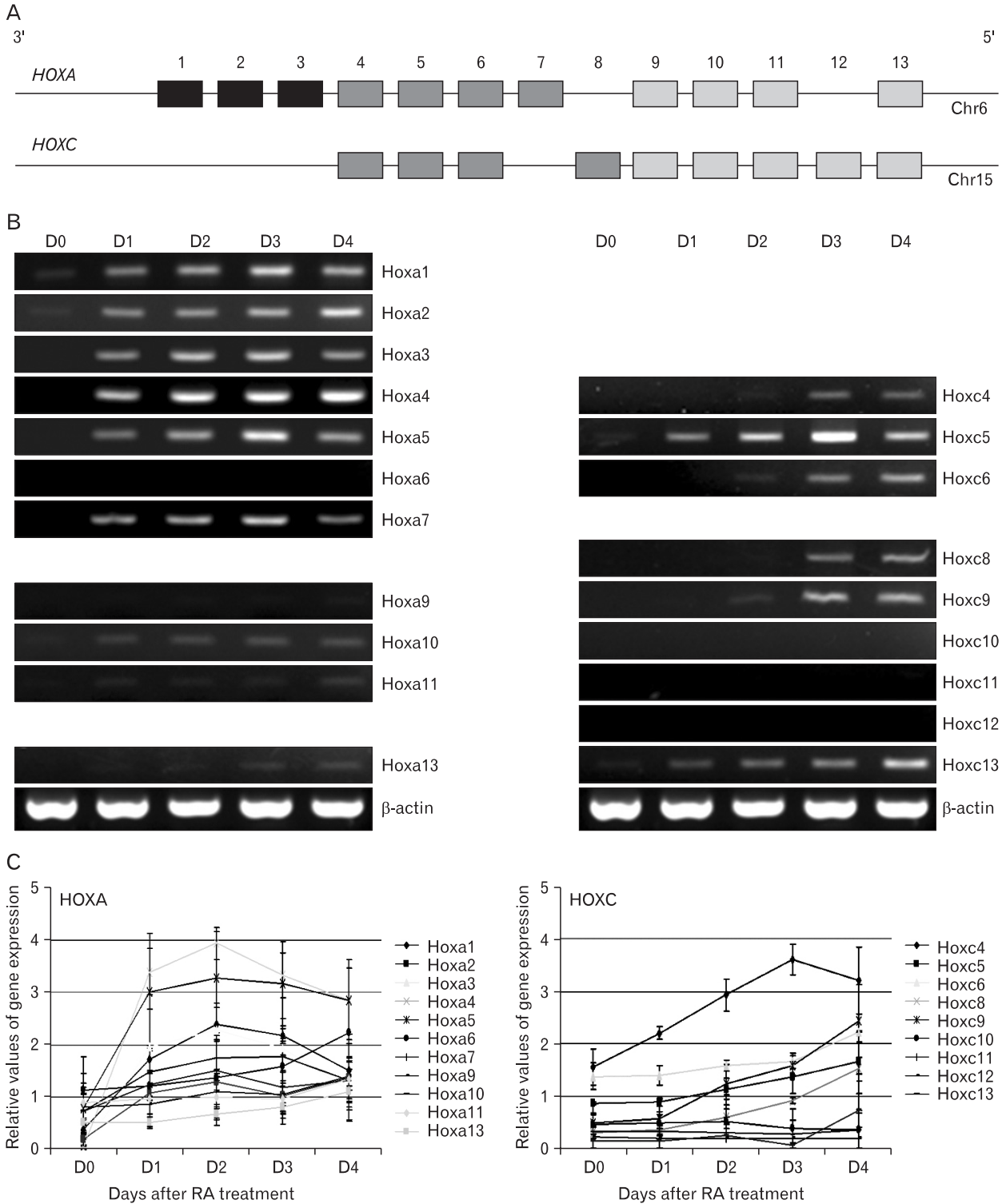
Fig. 1. Expression pattern of *Hoxa* and *Hoxc* cluster genes following RA treatment. (A) *Hoxa* and *Hoxc* gene organization in each cluster. (B) F9 cells were cultured with RA for 4 days. Total RNAs were isolated each day from Day 1 to Day 4 (D0: control cells with no RA treatment), and then RT-PCR was performed with each *Hox* primers. β-actin was used as a positive control for amplifiable cDNA. Data are representative of one of three replicate experiments. (C) The relative expression level of each *Hox* gene was determined by using Multi Gauge software. The amount of each RT-PCR product was normalized with that of β-actin to obtain relative intensities. Each point represents the average of three separate experiments. Error bars represent SEM.

used as a template. PCR was performed by using hTaq polymerase (Solgent, Seoul, Korea) under the following conditions: initial denaturation for 3 min at 95ºC, and then 27 cycles of 95ºC for 40 sec, 58ºC for 20 sec and 72ºC for 30 sec. We introduced a touchdown PCR method for detection of genomic band from combination of aP and cP series of primers. PCR cycle consisted of initial denaturing at 95ºC for 3 min, 10 cycles of touchdown PCR wherein the beginning annealing temperature of 65ºC was decreased 0.5ºC per cycle for 10 cycles with extension at 72ºC for 30 sec, denaturation at 95ºC for 1 min, and the following 40 cycles were at 95ºC for 1 min, 60ºC for 20 sec and 72ºC for 30 sec. The resulting PCR products were analyzed by conventional agarose gel electrophoresis.

Results

Sequential *Hox* gene expression in RA-treated F9 cells

We first examined here the expression profile of *Hox* genes following RA treatment. To investigate the expression patterns of *Hox* genes located in *Hoxa* and *Hoxc* cluster, F9 cells were treated with 5×10^{-7} M RA for 4 days. None of *Hox* genes showed an expression detectable in untreated F9 cells. Whereas *Hoxa* cluster genes, especially *Hoxa1* to *Hoxa7* genes which are located at the 3' region of their loci, are rapidly induced by 24 hr treatment with RA. *Hoxa9* to *Hoxa13* genes,

near the 5' end of the *Hoxa* cluster are only slightly induced after 3 days of RA treatment. Notably, most *Hoxc* cluster genes are lately induced at Day 3 and Day 4 after RA treatment (Fig. 1). These results demonstrate that the addition of RA to growing F9 cells induce *Hox* gene expression with gene-specific kinetics, especially *Hoxa* cluster gene was induced earlier than *Hoxc* genes. In *Hoxa* cluster, the phenomenon of spatial collinear expression, such that more 3' located genes are activated first, whereas more 5' located genes are expressed later, was partially actualized in current conditions.

Quality control of 3C templates by PCR

To determine whether chromatin conformation change is coupled with the gene expression in *Hox* cluster, we applied 3C method and analyzed physical contacts between DNA segments within *Hox* loci. First of all, the efficiencies of digestion and ligations were examined to validate whether the 3C templates reliably detect the chromosome conformation changes. The efficiency of digestion was tested by comparing PCR product from the primers D1/D2 annealing to the region spanning a restriction enzyme site of *Hin*dIII and the product from the primers D3/D4 annealing to the region lacking restriction enzyme recognition site within the amplified fragment (Fig. 2A). The result showed that the restriction enzyme digested all different set of templates with similar efficiencies. The efficiency of self ligation was examined by PCR using primers L1/L2. As shown in Fig. 2B,
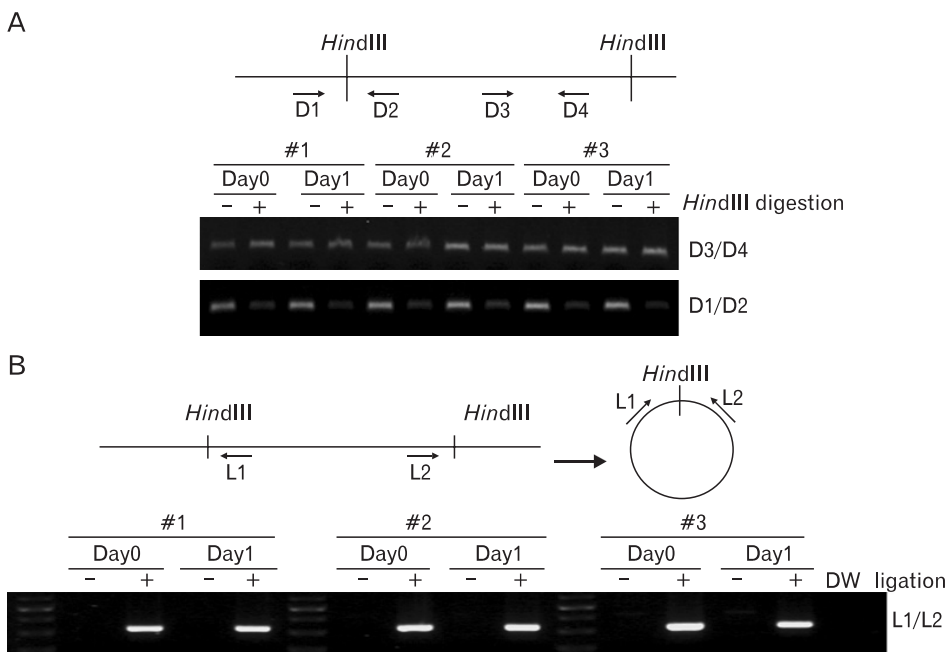

Fig. 2. PCR analysis of restriction digestion and ligation efficiency used for the evaluation of 3C template (A) To determine digestion efficiency, primer pairs D1/D2 and D3/D4 were used. When the *Hin*dIII restriction digestion was completed, D1/D2 primer pair gives a faint PCR band whereas D3/D4 primers generate a band whether DNA templates are undigested (−) and digested (+) with *Hin*dIII. (B) To determine the efficiency of self ligation, the primer L1/L2 was used. DNA was prepared before (−) and after (+) the ligation step. The 3C templates from Day 3 of RA treatment were also tested with same ways and obtained similar results (Data not shown).
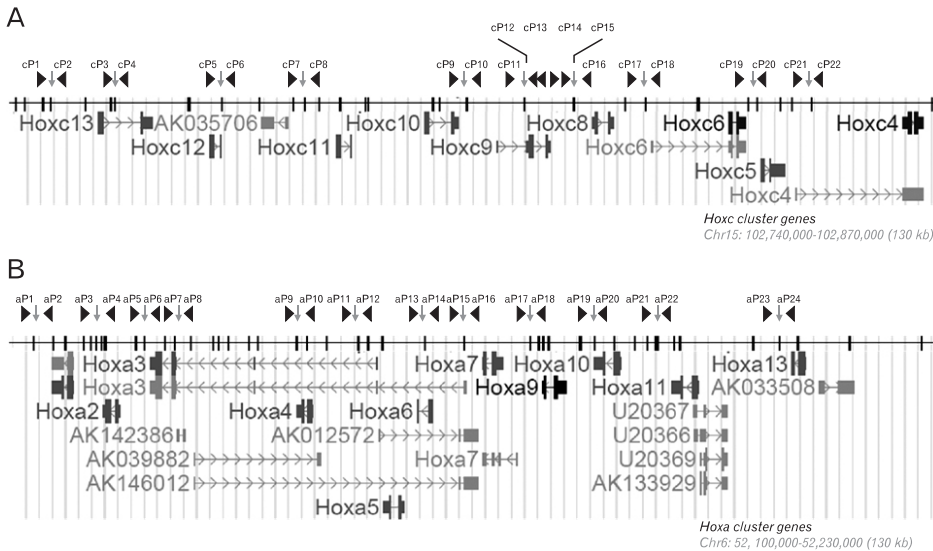
Fig. 3. Schematic presentation of the *Hox*c (A) and *Hox*a (B) loci showing the primer as well as *Hin*dIII regcognition sites. The UCSC Genes track includes both protein-coding and putative non-coding transcripts. The black bar depicts the *Hin*dIII recognition sites. The position of each primer we used for 3C analysis is indicated as arrowhead along with a serial number (cP1-cP22 and aP1-aP24 in *Hox*c and *Hox*a clusters, respectively). Each *Hox*c and *Hox*a region we analyzed covers 130 kb in mouse chromosome 15 and 6, respectively.

Table 1. Primers used in 3C PCR

| Hoxc			Hoxa		
Primer	Sequence (5' to 3')	Product size with cP12 (bp)	Primer	Sequence (5' to 3')	Product size with cP16 (bp)
cP12	TCT GCC CCT GGT CAG CTC AGT TTC	Anchor	aP16	GCA GCC GCC GCT GCT TTG CAA C	Anchor
cP1	GCA CTG TAA AGA GGA CTA TGG GTG	246	aP1	GGT CGC TTG TCT CTC AAA ATG C	254
cP2	CTT CGG AGG AAT GGA GGA ATG GAG	175	aP2	TCT ATG ACC TCC TAG AGC AC	283
cP3	CCA TAC ACC ACC TTC TCC TCA TTG	221	aP3	CCC TGC TCA GAT CTC AGA GCC	269
cP4	CTC TAG GCC TGA GTC CTT ACC TAC	175	aP4	GGT GAC TTA CCG TGA GAA ACT GC	207
cP5	GGT CAA GAT TTG GTT CCA GAA CCG	156	aP5	CTC TGC CAG CCA GTC AGC GGG C	183
cP6	TGG CGT TGT AAG CGG GAC TAT GTC	108	aP6	CCC GGA ATT CAG CAT CTG CC	266
cP7	CTC TGG CGA ATC TTC CTG TAA CTC	218	aP7	CTT GTC GGC ACT GAA GCC AC	295
cP8	GCC TTC TGC CTG CAC ATT GAC TTC	141	aP8	CCA GGC TTC TCC GAC TTG AG	262
cP9	ATC TTT GAT GGC ACT TAG GGG CGT	135	aP9	CTG CGC ACT AGG TCA AGT ATC	191
cP10	AAC ATG GGT GTC TAG ACC ACA GCG	198	aP10	CTG GGG CAC AGC GTT GGG GAG	273
cP11	CTC CAC TTC TTC AGG CTG GCA AAG	203	aP11	GGC TTA GCT CCT CAG ACA GG	222
cP13	CTT GAC GTA ATC TGT CTC TGT CGG		aP12	TAC CCC TTT GTC AGT AGG AG	315
cP14	TTT CTC CCA ACT TGC CTT CGG CTC	208	aP13	GCC AGG TGT GAG GTT CAG GC	274
cP15	GCA TTG GAA CAA GTG AAC GCG AGG	147	aP14	CCT CCA GAA GGT AGC ATA GGC	266
cP16	TTG ATG CCT GCC CTC TTT CGT CTC	209	aP15	GTG ACG TGT TTG GAC TGT GG	304
cP17	GGG GCT GCT CTA GAC TAA CAC AAC	170	aP17	CGG AGG CAG ACA GAG CAA AC	259
cP18	CCG GCT GCT CTA GAC TAA CAC AAC	213	aP18	GTT GAC TGT CTT CTA AGC CGG G	252
cP19	CTC CAG TCA CAT CCC AGA GAG TAC	184	aP19	GAA GGT CAG CCT GCC GTC AG	283
cP20	TAC CTC CAG AGC CTT CCA AAC TTC	208	aP20	CGC CAC CAC CCA CTC TGG TTT G	239
cP21	TTT GAG CCA CAG CTG CAG CAG TTC	172	aP21	CCC AGC ACA GCT GAG TTC CT	222
cP22	CTA CCA TCA TCT CTT TTG GGA CCC	169	aP22	GCC CGT TTG CCA CCC TCC AAC	240
			aP23	GTC AGA GCT CAG TTC CCT TG	165
			aP24	TGA CAG GAC TAG GAA AGG CC	253
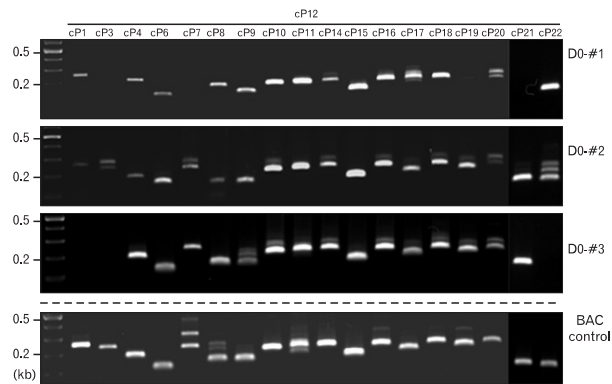
Fig. 4. Identification of intrachromosomal interaction of RA-untreated (D0) samples at *Hoxc* loci. 3C-PCR was performed with the primers indicated. The primer cP12 was used as an anchor. 3C DNA templates of 250 ng were used per each PCR. This experiment was performed three times (D0-#1, D0-#2, D0-#3) for validation of this method.

Fig. 5. Identification of intrachromosomal interaction of RA-treated (D3) samples at *Hoxc* loci. 3C-PCR was performed with the primers indicated. The primer cP12 was used as an anchor. 3C DNA templates of 250 ng were used per each PCR. This experiment was performed three times (D3-#1, D3-#2, D3-#3) for validation. Compare to those of Fig. 4, many PCR bands were disappeared indicating the disappearance of intrachromosomal interaction.

the PCR bands were detected only in ligated samples. Our PCR analysis also proved there was no preference for any particular region both in digestion and ligation.

Dynamic *Hoxc* chromatin architecture upon gene expression

To determine whether the activation of gene expression is accompanied by chromosome conformational changes, we performed 3C analysis in *Hox* loci. The positions of primers used in 3C-PCR are presented in Fig. 3. All of the primers were designed at a site that is ~50 to ~200 bp from the *Hin*dIII recognition site. The primers P12 in *Hoxc* cluster (cP12) and P16 in *Hoxa* cluster (aP16) were used as an anchor to perform PCRs together with other test primers in each locus. Primer sequences and the predicted size with anchor primers are listed in Table 1. Each primer was tested with BAC DNA containing whole *Hoxa* or *Hoxc* cluster genes after *Hin*dIII digestion and random ligation. With the combination of cP12 and test primers residing in *Hoxc* loci, many PCR products were generated from a large number of primer pairs in untreated D0 samples (Fig. 4). 3C-PCR was performed with replicates from each condition and comparable results were obtained, although the reproducibility for long-range interactions was worse than the short-range ones. Interestingly, these all possible interactions, except few (such as cP11, cP14-16), were undetectable in RA-treated Day 3 samples (Fig. 5), when most of *Hoxc* cluster genes are overall induced (Fig. 1). Pointed out the fact that the bands that were detected in Day 3 samples are originated from self-ligated or
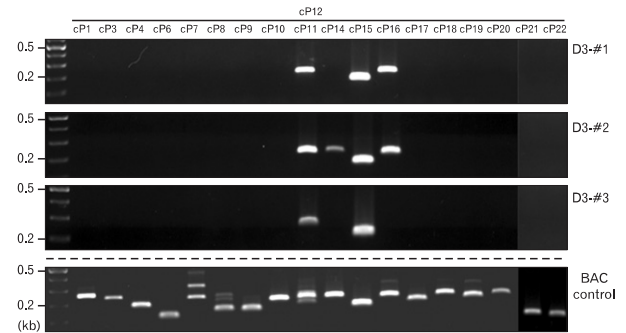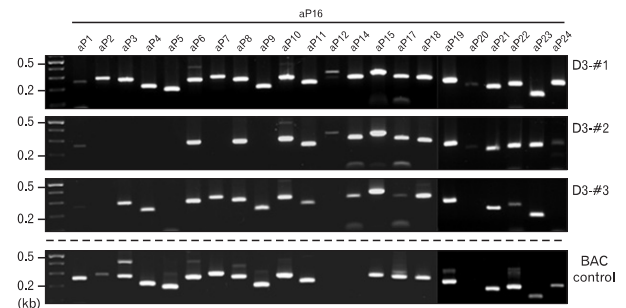

Fig. 6. Identification of intrachromosomal interaction of RA-treated (D3) samples at *Hoxa* loci. 3C-PCR was performed with the primers indicated. The primer aP16 was used as an anchor. 3C DNA templates of 250 ng were used per each PCR. This experiment was performed three times (D3-#1, D3-#2, D3-#3) for validation.

uncut DNA, we conclude that intrachromosomal interactions in *Hoxc* loci at Day 3 of RA treatment are quite rare. PCR bands from BAC control template ensured all possible ligation and similar efficiency of amplification from each primer pair (Fig. 4, 5). Unquestionably, the quality of Day 3 sample was validated by doing PCR with primer pairs for *Hoxa* loci (Fig. 6). *Hoxa* loci, in contrast to *Hoxc* loci, revealed similar interaction profiles in both untreated and RA-treated Day 1 samples (data not shown), implying that chromosome conformation at *Hoxa* loci was constant regardless of *Hoxa* gene induction. These results suggest that the spatial organization of the *Hoxc* loci, but not of the *Hoxa* loci, is dynamically changed, which was accompanied by *Hoxc* gene expression by RA.

Discussion

The establishment and maintenance of differential gene expression pattern of *Hox* gene is of fundamental importance during development or adult tissue maintenance. Recently there has been a great deal of interest in analyzing physical contacts between control DNA elements and target genes as an attempt to explain how spatial chromatin organization is critical in highly regulated gene expression. Considering the fact that appropriate spatial and temporal regulation of gene expression is actualized by the combined action of multiple regulatory elements which are dispersed throughout large genomic regions, the finding and characterization of the genomic elements which tune differential gene transcription through physical interactions with the target genes will provide new insight into a three-dimensional view of cell regulatory network. The present study reinforces the notion that the genome's spatial organization has great potential for gene regulation.

3C technique potentially allows identification of physical interactions between any chromatin segments. Therefore, it has been successfully applied to demonstrate long-range interactions, for example between transcriptional regulatory elements and distal target genes (Tolhuis *et al.,* 2002; Murrell *et al.,* 2004; Spilianakis *et al.,* 2004). In this study, we have generated 3C templates and proved their quality by comparing PCR products in two important steps of analysis, that is, restriction endonuclease digestion and ligation, between normal experiments with mock controls (un-digested/un-ligated samples). Together with the results for the quality control, reproducible detection of chromosomal interactions in three independently cross-linked samples was good signs to prove the validity of our samples. Although we were unable to detect interchromosomal interactions between *Hoxc* and *Hoxa* loci with the defined testing primers we used in this experiment (Data not shown), we cannot exclude a possibility of interchromosomal interactions occurred in other regions where we did not tested here.

Our 3C results showing abundant intrachromosomal interactions at *Hoxc* loci in RA-untreated samples, but not in Day 3 of RA-treated samples, implies that the compact chromatin structure was formed at *Hoxc* loci when the genes are transcriptionally inactive so that they interact frequently with neighboring fragments. Perhaps the chromatin would have turned into loose to facilitate transcription process at Day 3 after RA treatment, resulting in less interaction among

DNA within *Hoxc* loci. Previous studies on the genome-wide scanning of *Hoxb1*-associated loci in mouse ES cells using 3C-based technique demonstrated that the proportion of interchromosomal interactions between *Hoxb1* and the rest of the genome increased after its induction, while interactions with distal intra-chromosomal loci become less frequent (Würtele *et al.,* 2006). These results were compatible with FISH data showing decondensation and repositioning of *Hoxb1* outside of its chromosome territory during its expression (Chambeyron *et al.,* 2004). In addition, a study using THP-1 human myelomonocytic cell lines demonstrated that the repression of *Hoxa9*, *10*, *11* and *13* expression during cell differentiation is associated with the formation of distinct contacts between the genes and with an overall increase in chromatin packaging (Fraser *et al.,* 2009). Taken together with the results of previous studies, the current findings on dynamic chromatin organization at *Hoxc* loci strongly suggest that spatial organization of *Hox* loci plays a role in gene regulation.

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