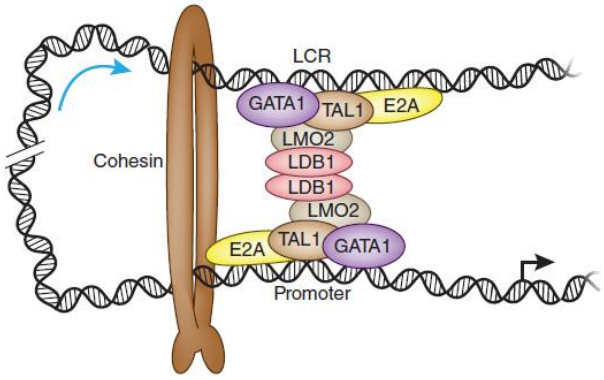
The role of eRNA in chromatin looping

Introduction

Enhancers are *cis*-acting DNA regulatory elements that activate transcription of target genes[1,2]. It is estimated that 400 000 to >1 million alleged enhancers exist in the human genome, indicating a high complexity of enhancer involvement in gene regulation[3]. The β-globin locus and its enhancer region, the locus control region (LCR), is a well-studied example of enhancer-promotor interaction[4]. To activate transcription, a change in chromatin conformation brings the LCR and the target promoter in close enough proximity to interact[1,4]. The resulting structure is a chromatin loop[4]. As illustrated in **Fig. 1**, several DNA-interacting and effector proteins are involved in mediating the chromatin loop, including the ring-like cohesin.

Figure 1[4]
The mammalian chromatin loop structure
This model illustrates the conformation of looping chromatin and some of the factors involved in DNA-binding and structure of the β-globin LCR and its target promoter. The cohesin ring-like structure is believed to hold the LCR and promoter together during interaction.



Some noncoding RNA (ncRNA) is transcribed from the DNA sequence of enhancer regions and such transcripts are referred to as enhancer RNAs (eRNAs)[1]. It is known that ncRNA is a highly functional class that possesses enzymatic activity, structural roles and transcriptional functions[3]. It is, however, yet unknown what the specific functional role of eRNAs is.

Several models have been proposed to explain the function of eRNAs, and there is evidence that at least some eRNAs contribute to enhancer function[3]. eRNAs that contribute to enhancer activity are likely to interact with other effector molecules, and whether this happens via a *cis* or *trans* mechanism is unclear[3]. Some RNA species are involved in protein-RNA complex formation and this supports some of the models of cell-type specific eRNA function[3]. eRNA may help facilitate or maintain the spatial interaction between enhancer and promoter that is established during chromosomal looping[5].

Enhancers involved in chromatin looping with promoters of protein-coding genes have increased expression of eRNAs, and this further indicates potential eRNA involvement in the process of chromatin loop formation[3]. Knockdown of eRNAs results in reduced enhancer-promoter interaction, decreased transcription of protein-coding genes, and decreased loop formation between enhancers and genes[2,3]. This further supports a direct role of eRNA in loop formation between enhancers and target genes, and leads naturally to the question:

How is eRNA involved in chromatin loop formation?

It has been known for a long time that eRNA exists, but it has only been known for a few years that eRNA transcripts play a functional role in gene regulation, and are necessary for proper enhancer-promoter loop formation[3,6]. In clarifying the role of eRNA in chromatin loop formation, this study will improve our understanding of the regulation of gene expression in homeostasis and development, and contribute to the expansion of treatments for various enhancer-related diseases, known as enhanceropathies, and maybe even specific types of cancer[3,4].

In human breast cancer cells, the bound oestrogen receptor causes increased transcription of eRNA on adjacent enhancers to upregulated coding genes[6]. In prostate cancer cells, the androgen receptor (AR) is a key factor in regulation of the cells. RNAi silencing of eRNA transcribed from enhancers of an AR-regulated gene causes a modest negative effect on proliferation of such cells[5]. Targeting specific eRNA with elevated transcription levels in specific types of cancer could be of particular interest to antisense oligonucleotide-based treatments. In order to design antisense therapeutics, it is critical to know the mechanistic role of eRNA, in order to optimize targeting and develop effective treatments.

Having established the relevance of determining the role of eRNA in chromatin loop formation, it is possible to propose a hypothesis for the mechanism by which it occurs.

Hypothesis

eRNA contributes to chromatin loop formation by linking proteins between enhancer and the target promoter during chromosomal looping.

Prediction

If this hypothesis is right, it will be possible to isolate eRNA with bound proteins known to interact with the β-globin LCR and the promoter of β-globin major gene during chromatin looping.

It is further possible that the transcriptional stage of locus activation, ranging from inactivated to fully transcribed, influences the contribution of eRNA to either loop formation or loop maintenance. This study will examine if eRNA can be isolated with bound proteins during different stages of β-globin locus activation and chromatin structure.

Experimental strategy

Cell line and transcriptional stages

Since long-range promoter-enhancer interactions are very well studied in the mouse β-globin locus[4], this locus is suitable for studying factors involved in chromatin looping. In murine etrythroleukaemia cells (MEL) from mice, the β-globin major gene can be transcriptionally induced by treating the cells with HMBA, (10-[(3-Hydroxy-4-methoxybenzylidene)]-9(10H)-anthracenome)[1]. Chromatin looping of the LCR and β-globin major gene is induced and eRNA transcription from the LCR is increased upon HMBA treatment[1]. All of these properties make the MEL cell line optimal for this study.

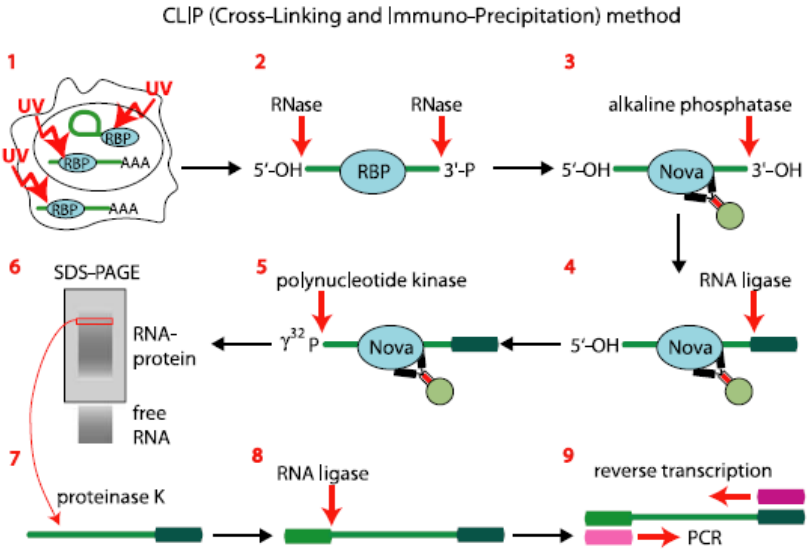
To activate transcription of the β-globin major gene, MEL cells with a density of 1.5 x 10^5/mL of will be treated with 5 mM HMBA for 24, 48 or 72 hours[1]. Three different transcriptionally activated locus stages are inducible depending on the duration of HMBA treatment of MEL cells. The locus is activated (eRNA transcription occurs, but no increase in transcription of the β-globin major gene[1]) after 24 hours (h) of HMBA treatment, after 48 h the locus is transcriptionally activated, and after 72 h the locus is fully transcribed[1]. These stages of activation will be examined, together with an inactivated stage (untreated cells), to determine if eRNA is associated with looping proteins in a stage-dependent manner. Untreated MEL cells will function as control cells with the purpose of revealing eRNA interactions with the looping proteins in uninduced cells.

At the end of HMBA treatment of the induced cells, the protocol by Ule *et al.* (2005) will be applied to conduct **c**ross-**l**inking and **i**mmuno**p**recipitation (CLIP) experiments. Purified RNA will subsequently be evaluated using RT-PCR and DNA sequencing.

The CLIP experiments

Several CLIP experiments will be conducted, one for each of the targeted proteins to immunoprecipitation. This is necessary to pinpoint potential eRNA interaction to the exact protein(s). The overview of the CLIP experimental procedure is shown in **Fig. 2**.

Figure 2[7]
CLIP experimental overview
1) *After HMBA treatment ends, the cells are UV-irradiated*
2) *Partial digestion of RNA and immunoprecipitation*
3) *Dephosphorylation of 3'-end*
4) *Linker ligation by RNA ligase*
5) *Addition of radioactive phosphate to 5'-end* ***6)*** *SDS-PAGE electrophoresis and transfer to nitrocellulose*
7) *Excised protein-RNA complexes digested by proteinase K to free RNA*
8) *Protein-free RNA is ligated to 5'-end RNA linker* ***9)*** *RNA is amplified by RT-PCR with DNA primers complimentary to RNA linkers, followed by DNA sequencing.*



The CLIP experiment allows *in vivo* cross-linking of live intact cells using UV irradiation (Fig. 2, step 1). After HMBA treatment, UV-irradiation is applied to cross-link proteins to nucleic acid. UV-irradiation at 254 nm does not result in cross-linking of protein-protein interactions, and the covalent bonds formed are irreversible[7].

By purifying specific protein-RNA complexes using CLIP, followed by SDS-PAGE separation, the vast majority of non-specific RNA and DNA contamination that co-immunoprecipitate is removed (Fig. 2, step 6)[7]. To completely eliminate DNA contamination, there are steps of DNAse reactions[7]. The protocol applied also includes steps (Fig. 2, step 4 and 8) where RNA linkers are ligated to first protein-bound and then free RNA. These linkers are complementary in sequences to the primers used in the subsequent RT-PCR experiment.

The proteins

The partial RNA digestion is followed by immunoprecipitation of proteins cross-linked to RNA (Fig. 2, step 2). The proteins known to be directly or indirectly involved in chromatin looping of the mammalian β-globin locus, including the LCR, can be categorized into three groups[8]. These proteins, listed below, will be subjected to immunoprecipitation in the CLIP experiments:

Erythroid transcriptional activators: EKLF, GATA1, NF-E2[8], TAL1, E2A, and LMO2[9]. These transcriptional activators are involved in DNA-binding to the LCR and β-globin gene promoters, and involved in chromatin structural changes such as nucleosome remodeling[8].

Co-factors: Ldb1, Brg1[8] and FOG1[2]. These proteins are involved in chromatin loop formation in the β-globin locus and form complexes with the transcriptional activators[8]. The Mediator complex, which interacts with enhancer-bound factors[4] and ncRNA in chromatin loop in HEK293 cells[10], and CTCF, which mediates long-range chromatin looping[8,11], are also included in this study.

Cohesin subunits: Rad 21, SMC1 and SMC3[6,8]. These subunits are known to participate in chromatin loop formation in the β-globin locus[8], and it has been shown that cohesin can interact with several eRNAs not associated with the β-globin locus[6].

Antibodies and eRNA evaluation

The antibodies (Abs) applied to immunoprecipitate the proteins are polyclonal, of isotype IgG, and pre-attached to beads before being added to the lysates[7]. The use of polyclonal Abs is preferred since the proteins of interest will be involved in multi-protein complexes formed during chromatin looping. Potential epitopes can be within these interaction-domains, hidden from Ab recognition[9]. Polyclonal Abs allow targeting of several epitopes on each of the proteins to avoid this potential problem[12,13].

An important preliminary control experiment, to prevent false positives, is to test the specificity of the Abs to the proteins that are immunoprecipitated[7]. This can be done using Western blot with buffers that retain sufficiently high-order protein structure to allow the primary Abs used in immunoprecipitation to recognize conformational epitopes[13]. Secondary Abs conjugated to an enzyme are then added to visualize the primary Abs.

Each polyclonal selection of Abs specific to each of the protein targets will be evaluated as described. These specificity experiments will be carried out in uninduced and induced MEL cells to evaluate if the induced activated stages (24 h, 48 h, and 72 h of HMBA treatment) affect Ab specificity differently.

To evaluate the purified RNA by the CLIP procedure, RT-PCR will be applied with DNA primers complementary to the ligated RNA linkers[7]. This allows amplification of all cross-linked RNA in samples into DNA products[7]. One important control experiment is the reverse transcriptase (RT) control, where no RT is added to the sample to evaluate DNA contamination in the purified RNA samples. The DNA product from RT-PCR will subsequently be purified and sequenced to assess if it is derived from the β-globin LCR sequence.

Possible outcomes

Positive result

eRNA from the β-globin LCR could be cross-linked to the immunoprecipitated proteins in induced MEL cells. LCR derived eRNA interacts with proteins involved in chromatin looping during active transcription of the β-globin major gene. A possible function of eRNA is to contribute to protein-interactions in loop formation or loop structural maintenance. This depends on whether eRNA cross-linking turned out to be transcriptional stage-specific. If eRNA is involved in loop formation, it would be expected that eRNA cross-link after 24 h of HMBA treatment, and maybe after 48 h. If eRNA is involved in loop maintenance, eRNA would be expected to cross-link after 72 h of HMBA treatment, and maybe already after 48 h.

This evidence supports existing models suggesting that eRNA has a mechanistic role in mediating the spatial interaction between enhancer and promoter. It is shown that eRNA works in a specific enhancer-promoter relation, and that eRNA interacts locally at the locus from where it is transcribed, but this does not rule out a globally functional role.

This study provides a better mechanistic understanding of eRNA function, which is important to disease understanding of enhanceropathies and treatment development. RNAi knockdown experiments and fluorescent probes could further be applied to evaluate if this LCR-eRNA has a function restricted to the β-globin locus, or if an eRNA-knockdown effect could be traced elsewhere in the nucleus. If a sub-group of the examined proteins were interacting with eRNA, and some proteins were not, this would provide further insight into the role of eRNA in the chromatin loop.

Negative result

If no eRNA is detectable when CLIP experiments are performed, then we may conclude that eRNA does not interact with proteins involved in chromatin looping during induced active transcription of the β-globin major gene. In this case, it is possible that this eRNA may contribute to enhancer function of the LCR, but this experiment does not support models that suggest eRNA is involved in mediating a spatial interaction between enhancer and promoter during chromatin looping.

This study provides evidence that support models of eRNA function that suggest different roles of eRNA than examined in this study. However, the involvement of eRNA in chromatin loops cannot be ruled out by this single experiment, it could further be examined whether the role of the eRNA is enhancer-specific, but also if the role varies between tissue-types and species.

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