Long-range chromatin regulatory interactions *in vivo*

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Communication between distal chromosomal elements is essential for control of many nuclear processes. For example, genes in higher eukaryotes often require distant enhancer sequences for high-level expression. The mechanisms proposed for long-range enhancer action fall into two basic categories. Non-contact models propose that enhancers act at a distance to create a favorable environment for gene transcription[1–3], or act as entry sites[4] or nucleation points[5] for factors that ultimately communicate with the gene. Contact models propose that communication occurs through direct interaction between the distant enhancer and the gene by various mechanisms that 'loop out' the intervening sequences[6–13]. Although much attention has focused on contact models, the existence and nature of long-range interactions is still controversial and speculative, as there is no direct evidence that distant sequences physically interact *in vivo*[14]. Here, we report the development of a widely applicable *in situ* technique to tag and recover chromatin in the immediate vicinity of an actively transcribed gene. We show that the classical enhancer element, HS2 of the prototypical locus control region (LCR) of the β-globin gene cluster, is in close physical proximity to an actively transcribed *HBB* (β-globin) gene located over 50 kb away *in vivo*, suggesting a direct regulatory interaction. The results give unprecedented insight into the *in vivo* structure of the LCR–gene interface and provide the first direct evidence of long-range enhancer communication.

We developed a modified RNA fluorescence *in situ* hybridization (FISH) method called RNA TRAP (tagging and recovery of associated proteins) to tag and analyze chromatin *in situ* at the site of a transcriptionally active mouse β-globin gene (Fig. 1). The TRAP protocol involves targeting horseradish peroxidase activity to the primary transcripts associated with an actively transcribed gene. The localized horseradish peroxidase then catalyzes the covalent deposition of a biotin tag on chromatin proteins in the immediate vicinity of the gene. Confocal microscopy and image deconvolution showed that biotin deposition was most intense in a central core region having a radius of ≤120 nm (Fig. 2). Shells of less intense deposition surrounded the core, and deposition lessened to background levels at points 300–500 nm from the epicenter.

The four mouse β-like globin genes require the distal LCR for high-level expression in erythroid cells[15]. The LCR consists of six DNase I–hypersensitive sites (HS) located 35–60 kb upstream of

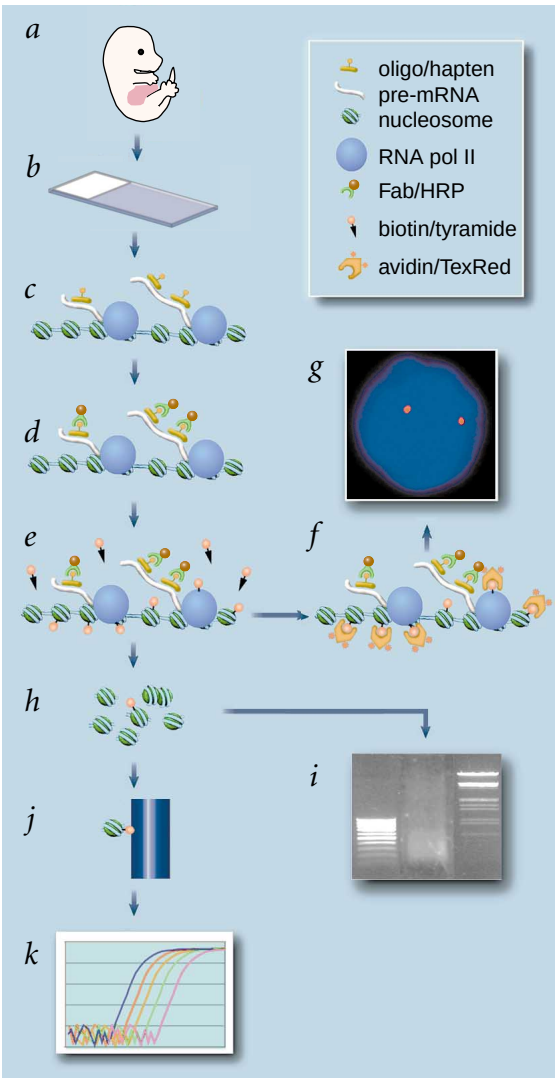

Fig. 1 RNA TRAP. Livers were collected from E14.5 mouse embryos (***a***), disrupted, spread on poly-L-lysine coated slides (***b***) and fixed with formaldehyde. Gene-specific intron probes (oligonucleotides) labeled with digoxigenin were hybridized to primary transcripts (***c***). A Fab fragment conjugated with horseradish peroxidase was added to localize horseradish peroxidase activity to the site of transcription (***d***). Biotin-tyramide was added, which, on contact with horseradish peroxidase, becomes a highly reactive radical intermediate that covalently attaches to electron-rich moieties (principally tyrosines) in the immediate vicinity (***e***). Targeting of biotin-tyramide deposition was checked by adding an Avidin D–Texas Red–conjugate (***f***) and examining the slide using fluorescence microscopy (***g***). Cells were removed from glass slides and fragmented by sonication (***h***), and chromatin fragmentation was monitored on agarose gels to ensure an average DNA size of approximately 400 bp (***i***, center lane; left lane, 100 bp ladder (up to 1,000 bp); right lane, λ *Hin*dIII/*Eco*RI). Cells were purified by affinity chromatography on a streptavidin–agarose column (***j***), and DNA sequences across the *Hbb* locus were quantified in affinity-purified versus input chromatin by PCR or slot blot analysis (***k***).

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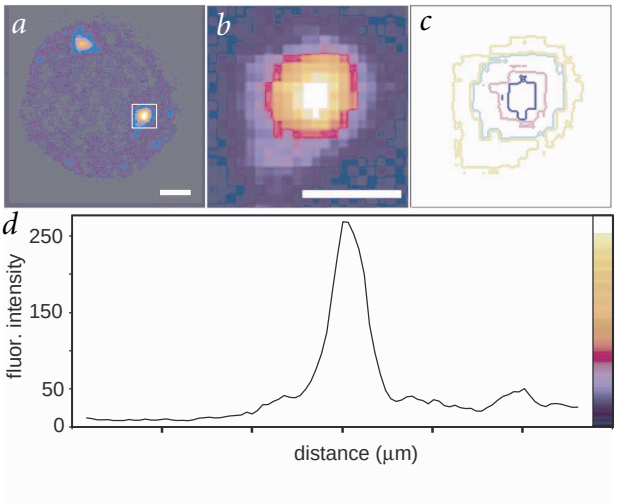

Fig. 2 Measurement of the spread of biotin deposition. ***a***, Pseudo-color median confocal section through typical RNA TRAP signal stained with Avidin-D conjugated to Texas Red. Scale bar = 2 μm. ***b***, Enlargement of boxed area in *a*. Scale bar = 1 μm. ***c***, Contour plot of signal shown in *b* showing shells of decreasing labeling density. ***d***, Intensity plot through signal shown in *b* with pseudo-color look-up table on right.

the *Hbb-b1* gene. We reasoned that if contact between factors bound to the gene and LCR cores was required for high-level transcription, then large amounts of biotin would be deposited on the LCR chromatin *in situ*, owing to its proximity to the active gene located in the central core of intense labeling. As a result, the LCR would be significantly enriched in the affinity-purified chromatin.

We isolated livers from embryonic day (E) 14.5 mouse fetuses in which only the adult-type *Hbb-b1* and *Hbb-b2* were expressed, and carried out an RNA TRAP assay using probes directed toward the *Hbb-b1* primary transcript. We measured enrichment of various sequences across the *Hbb* locus and the neighboring olfactory receptor gene cluster by quantitative real-time PCR. The greatest enrichment that we observed (20-fold) was near the transcription termination site of *Hbb-b1*, consistent with the position of the *in situ* probes in intron 2 near the 3′ end of the gene (Fig. 3*a*). Enrichment dropped off sharply upstream of *Hbb-b1* in the area of the developmentally silenced genes *Hbb-y* and *Hbb-bh1*, which were only slightly enriched relative to background levels. Notably, we found that HS2 and, to a lesser extent, HS1 and HS3 of the LCR were enriched.

We assumed that enrichment of any sequence relative to the gene would generally be due to both proximity and duration of proximity. But we could not, on the basis of this experiment, exclude the possibility that HS2 was enriched owing to preferential labeling of a particular local chromatin structure or factors bound there. The fact that other hypersensitive sites in the LCR (HS4, HS5 and HS6) and the downstream 3′ HS1 (which is closer to *Hbb-b1* than is HS2) were not markedly enriched suggests that there was no preferential labeling of areas of hypersensitive or open chromatin. To discount the possibility that these results were caused by preferential biotin deposition in certain areas (for example, in modified chromatin), we designed and carried out a control, random TRAP experiment. We omitted the intron probe during the FISH stage of the protocol, causing biotin to be deposited randomly across the genome such that any bias for certain sequences would be apparent in the analysis of the affinity-purified material. The results showed that there was no preferential labeling of sequences in the *Hbb* locus (Fig. 3*a*), indicating that enrichment of HS2 in the *Hbb-b1*-directed TRAP experiment was not caused by a chromatin bias. Although we cannot formally exclude the possibility that preferential labeling occurs when horseradish peroxidase is localized to the *Hbb* locus, our results showing specific labeling patterns for each of the active genes (discussed below) suggest that this is unlikely.

These results indicate that HS1, HS3 and, in particular, HS2 were closer to *Hbb-b1* than were the intervening sequences. Indeed, the 15-fold enrichment of HS2 suggests that it was very close to the core of intense labeling over the gene. The levels of enrichment for HS1 and HS3 were lower than for HS2 but still higher than background levels, suggesting that HS1 and HS3 were more peripheral than HS2 or were only transiently associated with the gene. These results are notable given that the human equivalent of HS2 is the only region of the LCR with classical enhancer activity, meaning that it is able to enhance gene expression in a transient transfection assay[16]. Human HS1, HS3 and HS4 increase levels of gene expression only in stably transfected cells and transgenic mice[17,18], indicating that they function only when integrated into chromatin. This suggests a structural rather than enhancer functional role. A central role for

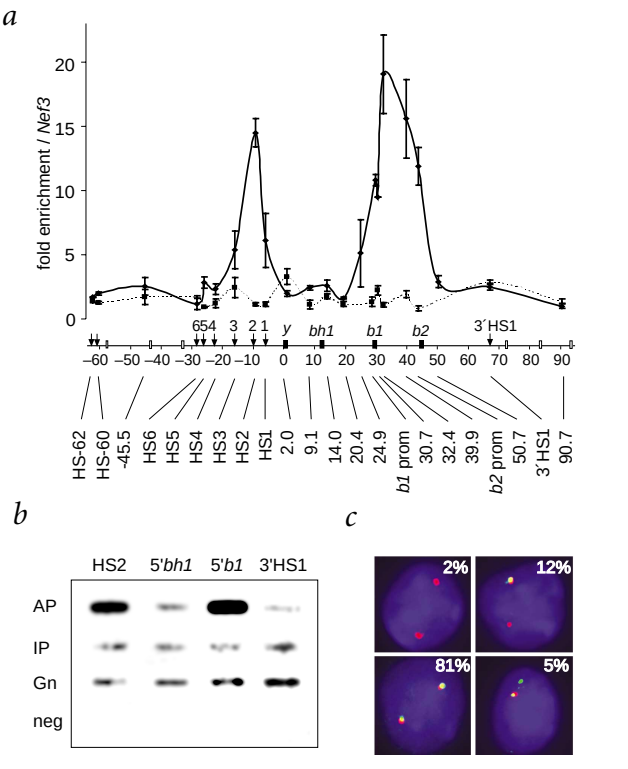

Fig. 3 Quantitative real-time PCR analyses of *Hbb-b1*-targeted RNA TRAP. ***a***, *Hbb-b1*-directed RNA TRAP (solid curve) assaying various sequences in the *Hbb* locus and the neighboring olfactory receptor gene locus using the primer pairs shown below the map. Error bars represent standard error. Enrichment was calculated from the ratio of affinity-purified chromatin to input chromatin, normalized to the gene encoding neurofilament 3, medium (*Nef3*; ref 24), which was used as a measure of background. Positions of the *Hbb* genes (black boxes), olfactory receptor genes (open boxes), LCR (HS1–HS6), 3′ HS1 and the hypersensitive sites at bp –61 (ref. 21; vertical arrows) are shown on the *x* axis. Results of a random RNA TRAP, quantified as above (dashed curve), showed that there was no preferential enrichment for any particular region of the *Hbb* locus. ***b***, Slot blot analysis of *Hbb-b1*-directed RNA TRAP. 0.3 μg of affinity purified (AP), input (IP) and mouse genomic (Gn) chromatin and 2.5 μg of human genomic DNA (neg) were loaded onto a nylon filter and hybridized with probes corresponding to the indicated regions of the *Hbb* locus. ***c***, Double-label RNA FISH using *Hbb-b1* (red) and *Hbb-b2* (green) intron probes. The four main transcriptional cell types are shown[20] along with the approximate relative percentages at which they are found in the fetal liver.

HS2 in transcription is also suggested by the fact that deletion of mouse HS2 from the endogenous locus results in a greater decrease in gene expression than does deletion of any of the other hypersensitive sites[19].

We repeated the *Hbb-b1* RNA TRAP assay three times and obtained similar results each time. We analyzed DNA from one of the *Hbb-b1* RNA TRAP assays by slot-blot assays with multiple probes, which yielded similar results (Fig. 3*b*). These data provide the first direct evidence that a distal enhancer is held in close physical proximity to an active gene that it regulates *in vivo*. Given the wealth of functional data showing transcriptional enhancement capabilities of the LCR and HS2 in particular, we suggest that HS2 is engaged in an intimate regulatory interaction with the transcriptionally active *Hbb-b1* gene.

We repeated the RNA TRAP assay with intron probes for *Hbb-b2*, located approximately 15 kb downstream of *Hbb-b1*. Both *Hbb-b1 and Hbb-b2* are transcriptionally active in erythroid fetal liver cells, and we showed by RNA FISH analysis that most *Hbb* gene clusters have simultaneous primary-transcript signals for both genes in *cis*[20] (Fig. 3*c*). In the *Hbb-b2*-directed affinity-purified chromatin, we observed a peak of enrichment over *Hbb-b2*, as expected (Fig. 4). In the LCR region, HS2 was highly enriched, suggesting that it was tightly associated with the active *Hbb-b2* gene. HS4 was also enriched compared with HS1, HS3, HS5 and HS6 of the LCR. These results suggest that HS2 is shared by both active genes and that HS1, HS3 and HS4 have unique positions relative to the genes. The fact that HS5 and HS6 were not associated with the site of transcription is compatible with the functional data showing that their deletion from the endogenous locus has no effect on steady-state RNA levels[21]. The enrichment of specific subsets of hypersensitive sites for each gene argues against the possibility that the results are due to preferential labeling of chromatin.

These results provide the first direct evidence that distant enhancer elements function in close proximity to the genes that they regulate *in vivo*, suggesting direct chromatin regulatory interactions (Fig. 5) and discounting non-contact models of long-range enhancer action. There are several possible models of the arrangement of the LCR–gene complex, ranging from a multi-faceted LCR holocomplex[11] to a specialized microenvironment or transcription factory[22].

The RNA TRAP technique we developed should be applicable to a number of other genes for rapid identification and study of interactions between distal elements regulating transcription. We are currently adapting the technique for use with DNA FISH (DNA TRAP), which should provide more definitive information on the structure of the LCR–gene complex and permit the study of interactions that are not directly involved in transcription. The FISH TRAP technique and adaptations of it will greatly increase our understanding of the role of higher order chromatin structure and chromosome folding in various nuclear processes.

Methods

RNA FISH TRAP. We removed livers from E14.5 fetal Balb/c mice and disrupted them in ice-cold phosphate-buffered saline (PBS). All experimental procedures using mice were carried out under a project license granted from the Home Office UK. The cells were spread on slides coated with poly-L-lysine and fixed in 4% formaldehyde, 5% acetic acid for 18 min at room temperature. We carried out subsequent slide washing, permeabilization, probe hybridization and post-hybridization washing as described[23]. Endogenous peroxidases were quenched in 0.5% H_2O_2 (in PBS) for 10 min, washed for 5 min in TST (100 mM Tris pH7.5, 150 mM NaCl, 0.05% Tween 20) and blocked as described[23]. We incubated slides with antibody against the Fab fragment of digoxigenin conjugated with horseradish peroxidase diluted 1:100 for 45 min at room temperature in a humidified chamber, washed them twice for 5 min each in TST and then

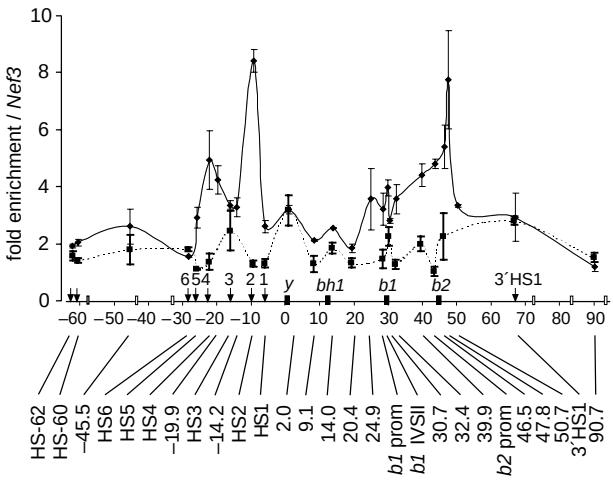

Fig. 4 Quantitative real-time PCR analyses of *Hbb-b2*-targeted RNA TRAP. *Hbb-b2*-directed RNA TRAP assaying various sequences in the *Hbb* locus (solid curve) as described for Fig. 3*a*. The greatest enrichment was observed in the area of *Hbb-b2* (compare with Fig. 3*a*, solid curve). Dashed curve shows results of a random RNA TRAP assay, as described for Fig. 3*a*.

incubated them for 1 min with 1:150 biotin-tyramide (NEN) under coverslips at room temperature. The slides were then quenched again in 0.5% H_2O_2 (in PBS) for 10 min, washed twice in TST for 5 min each and transferred to PBS for scraping.

We stained one slide with an Avidin/Texas Red conjugate for 45 min at room temperature. This slide was then washed, dehydrated, mounted and visualized by confocal microscopy.

We scraped the cells from the remaining slides, typically recovering approximately 25 million cells. The cells were spun down at 2,900*g* for 25 min, resuspended in 2 M NaCl, 5 M urea, 10 mM EDTA and sonicated for 200 s on ice (eight 25 s bursts with 1.5 min between bursts) using a Microson Ultrasonic Cell Disruptor set at level 5. We centrifuged the crude chromatin for 15 min at 10,000*g*, removed the supernatant containing the soluble chromatin, resuspended the insoluble pellet in 2 M NaCl, 5 M urea, 10 mM EDTA and sonicated it again. We centrifuged this solution again and then combined the two soluble fractions and dialyzed the mixture overnight at 4 °C against PBS. This procedure routinely yielded chromatin fragments with an average DNA size of 400 bp.

We set aside 10% of the soluble chromatin as the input and passed the rest over a streptavidin–agarose (Molecular Probes) affinity column. After binding took place, we washed the column with 3× 700 µl PBS, 2× 500 µl TSE 150 (20 mM Tris pH 8.0, 1% Triton, 0.1% SDS, 2 mM EDTA, 150 mM NaCl), 2× 500 µl TSE 500 (20 mM Tris pH 8.0, 1% Triton, 0.1% SDS, 2 mM EDTA, 500 mM NaCl) and 3× 700 µl PBS. We removed the beads from the

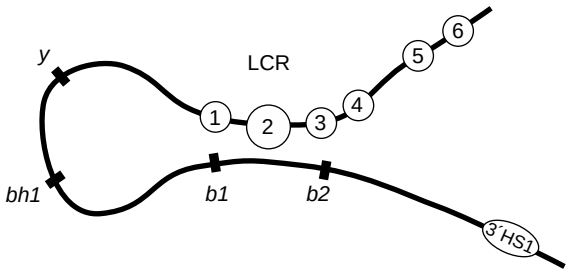

Fig. 5 Schematic representation of LCR–gene arrangement. LCR hypersensitive sites 1–6 are depicted as numbered circles. The *Hbb* genes are represented by black boxes. Our results suggest that HS1–HS4 of the LCR are in close proximity to the active *Hbb-b1* and *Hbb-b2* genes. It is not possible to determine the exact positions of the hypersensitive sites relative to the genes, but, given functional data, we suggest that HS2 in particular is involved in a direct regulatory interaction with *Hbb-b1* and *Hbb-b2*.

column, reversed the formaldehyde crosslinks and digested the protein components by overnight incubation at 65 °C with 200 μg ml^{-1} proteinase K while shaking vigorously. We then treated the samples with 20 μg ml^{-1} RNase A for 30 min at 37 °C and then 200 μg ml^{-1} proteinase K for 5 h at 37 °C, extracted them with phenol and precipitated them with ethanol using 20 μg ml^{-1} glycogen as carrier. We quantified DNA from the input fraction using a standard spectrophotometer and measured the DNA concentration of the affinity-purified fraction by picogreen quantification using the input chromatin as a standard.

Confocal microscopy and image deconvolution. We used a Biorad MRC1024ES confocal microscope and restored the images with the deconvolution software AutoDeblur (Autoquant) using the 'power acceleration' blind deconvolution algorithm. We used public domain software ImageJ to prepare the images.

Real-time PCR. Real-time PCR was carried out with an ABI PRISM 7700 sequence detector using 2× SYBR green PCR master mix (Applied Biosystems). For each primer pair, we generated a standard curve using 30 ng, 5 ng and 1 ng of the input chromatin, which was then used to quantify the enrichment of 1 ng of affinity-purified chromatin (all reactions were done in duplicate). We separated all PCR products on a 2% agarose gel to ensure that all reactions gave a single product.

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Competing interests statement

The authors declare that they have no competing financial interests.

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