Conserved noncoding sequences are reliable guides to regulatory elements

A 'working draft' of the human genome sequence is now available. Comparisons with the sequences of mouse and other species will be a powerful approach to identifying functional segments of the noncoding regions, such as gene regulatory elements. However, the choice of a species for most effective comparison differs among various loci.

In June 2000, the International Human Genome Sequencing Consortium announced a 'working draft' of the human genome. In this assembly, approximately 87% of the genome has been sequenced, with about 21% in finished form and 66% in draft form, exceeding the goals announced in 1998 (Ref. 1). (Updated information is available at http://www.ncbi.nlm.nih.gov/genome/seq) At the same time, Celera Corporation announced their first assembly of the human genome sequence. These are heady days for the sequencers. Even though a finished, high-quality sequence of the human genome will not be available for another year or more, the working draft sequence has so much information, sufficiently close to complete, that it marks a major milestone in modern biology. Moreover, the analysis of this information could lead to a revolution in the life sciences, as biologists begin to adapt to the limitations – and power – of having a finite (albeit large) number of gene products to consider[2]. Thus a pressing issue is how to proceed with functional analysis of this massive amount of sequence – currently greater than three billion base pairs!

Software for finding coding exons has reached a high degree of sophistication and accuracy[3], so most of the 3–5% of the human genome coding for protein will be identified in short order. Even after masking out the roughly 40% of the genome with interspersed repeats[4], analysis of the remaining half of the genome, the unique, noncoding portion, is a daunting task.

A powerful approach to finding functional segments in noncoding genomic DNA is comparison with other sequences. This approach has a rich history, dating at least to the discovery of bacteriophage promoter and operator sequences[5]. Comparison of long genomic DNA sequences between two species reveals conserved noncoding sequences (CNSs) (See Box 1 for definitions), and at the *HBB* (encoding beta-globin) and *BTK* (encoding Bruton's tyrosine kinase) loci, these have been demonstrated to be involved in the regulation of gene expression (reviewed in Ref. 6). Several recent papers compare human and mouse DNA sequences over long genomic segments (e.g. Refs 7–9), and in most cases they find that human–mouse sequence comparisons were useful both for accurately identifying exons and for finding candidate regulatory regions. The many CNSs observed should provide an impetus for a large number of experiments. However, it is not always clear what function to test for, or even which gene is a likely target of a proposed regulatory element. For instance, the distal major regulator of *HBA* (encoding alpha-globin) is found in the intron of a ubiquitously expressed gene of unknown function, located 40 kb away from the nearest globin gene[10].

A CNS affects expression of genes encoding interleukins

A recent report combines large-scale sequence comparisons with experimental tests to show that a CNS controls expression over a long range with high selectivity (Fig. 1). Loots *et al.*[11] determined the sequence of about a million base pairs from human chromosomal region 5q31, including the genes encoding five interleukins and potentially 18 other proteins. The orthologous region from mouse was also sequenced, with much of it in draft format. Sequence alignments revealed 90 CNSs; the longest, termed CNS-1, is located between interleukin 4 (*IL-4*) and 13 (*IL-13*). These genes, and also interleukin 5 (*IL-5*), are expressed in type 2 T-helper (T_H2) cells. All three cytokines, along with several other genes and CNSs, are contained in a 450-kb segment of human DNA in a recombinant YAC (Fig. 1). Loots *et al.* generated three lines of transgenic mice carrying this YAC; production of the human interleukins can be measured specifically without interference from the endogenous mouse interleukins. Importantly, they flanked CNS-1 with *loxP* sites so that it could be deleted by Cre recombinase after the YAC had integrated into the mouse genome. Although each line of transgenic mice carries the YAC at a different site of integration, the expression of the interleukin genes could be assayed with and without CNS-1 at each site, thus controlling for any position effects.

The results were dramatic. Deletion of CNS-1 reduced the production of IL-4, IL-13 and IL-5 about two- to threefold, specifically in T_H2 cells. A similar result was obtained in all three lines of transgenic mice. Thus CNS-1 affects not only adjacent genes but also a gene located about 120 kb away. Moreover, *RAD50*, a large gene lying between *IL-13* and *IL-5*, was unaffected by the deletion. Hence the effect of CNS-1 is exerted over a long distance, but in a highly selective manner, only regulating expression of cytokines specific to T_H2 cells. Although such selectivity has been seen in other systems[10], most known regulatory elements are closer to the target genes than to other genes. This latter notion might reflect an incomplete knowledge of regulatory elements and neighboring genes, and future studies might reveal more examples of regulatory sequences with this ability to skip over intervening genes. Clearly, the results of Loots *et al.*[11] demonstrate the power of the comparative genomic approach for finding distal regulatory sequences.

The reduction in IL-4 and IL-13 resulted from a decrease in the number of T cells expressing the genes; the amount of these cytokines produced in expressing cells was unaltered by the deletion of CNS-1. Thus CNS-1

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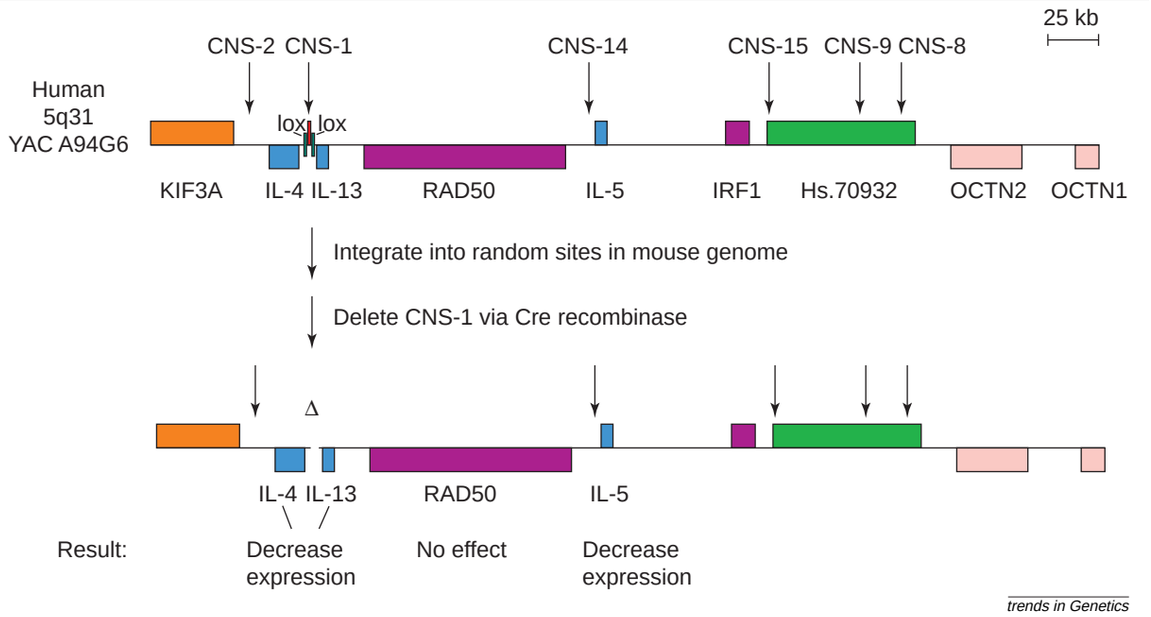
FIGURE 1. Deletion of a conserved noncoding sequence selectively reduces expression of cytokine genes over a long distance



Genes from human chromosomal position 5q31 located on YAC A94G6 are shown as boxes; those above the line are transcribed 5′ to 3′ to the right, those below the line are transcribed to the left. Genes encoding interleukins are colored blue; other genes are arbitrary colors. Conserved, noncoding sequences are shown as arrows. Sites (*loxP*) for directing deletion by the Cre recombinase are green boxes.

affects the probability that this chromosomal region will be transcriptionally active in a given T_H2 cell, but once the region is activated, CNS-1 is not needed for an appropriate level of expression. It is likely that an epigenetic process is affected, such as changes in chromatin structure, DNA methylation, or localization in the interphase nucleus. Explaining how such processes can be targeted to specific genes and not others over 120 kb is a major challenge. Models involving looping, tracking and linking have been proposed for the action of other distal regulators, such as the *HBB* locus control region, but experimental tests have yet to be devised that clearly distinguish between them[12].

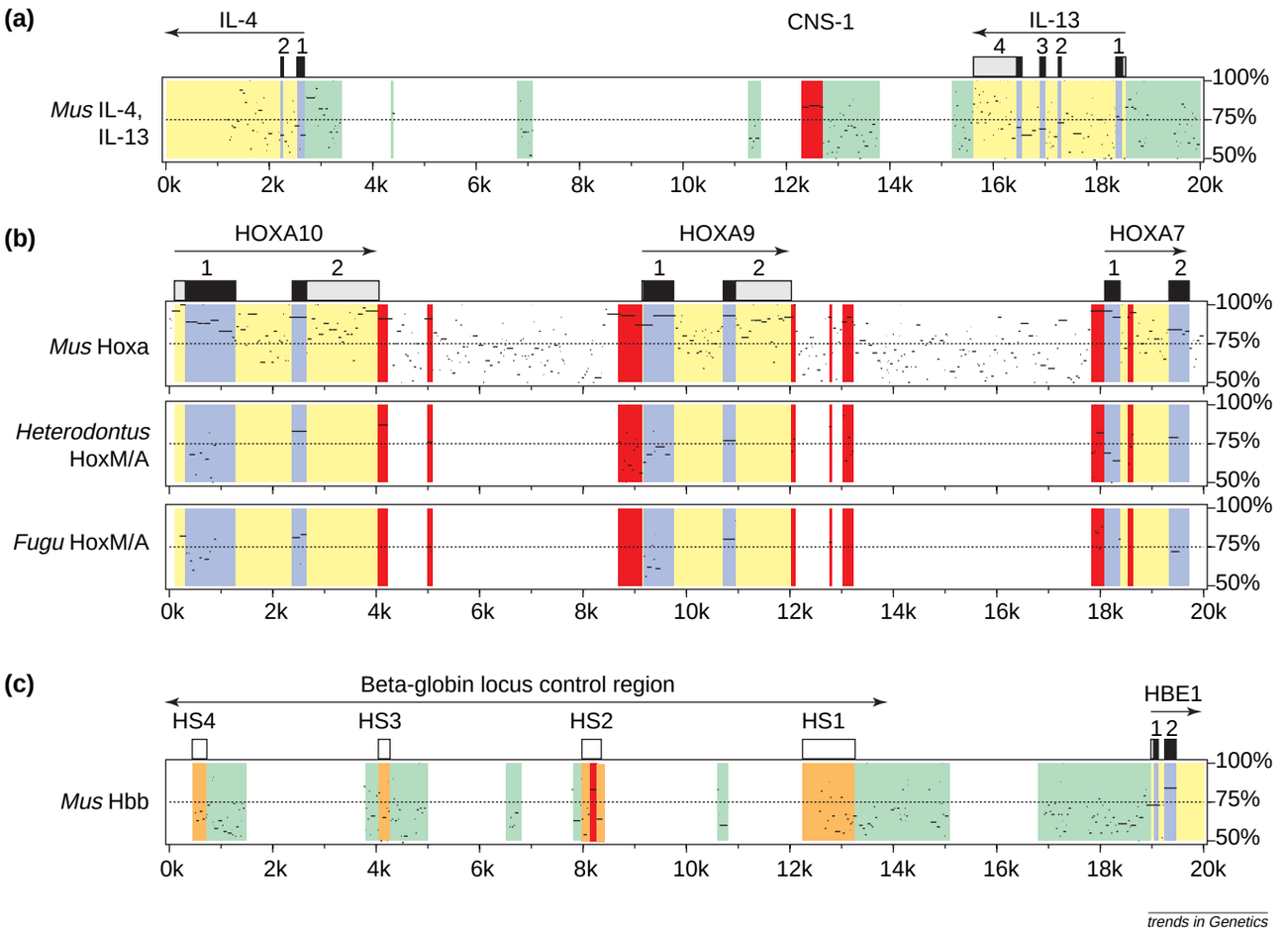
Different comparison species are needed to find functional CNSs at various loci

If one can extrapolate from the density of CNSs at 5q31, a total of 270 000 CNSs in the human genome might be predicted. Full exploration of these CNSs would require an enormous number of transgenic mouse experiments, doubtless exceeding any conceivable budget for such an enterprise. Indeed, high-throughput assays, such as the transgenic frog assay for vertebrate transcriptional enhancers[13], are being developed to allow rapid testing of the myriad candidate regulatory sequences. In contemplating how the results at 5q31 should impact work at other loci, two major issues come to mind. Are all these CNSs likely to be functional? And are the regulatory sites likely to be found within the CNSs? The most satisfying answers will come from experimental tests, but other information, such as additional sequence comparisons, is needed to direct experimentalists to the most critical regions.

To reduce the number of CNSs to test, one could compare sequences of species separated by a wider phylogenetic distance, as it is reasonable to assume that noncoding sequences that have survived selection for a longer period of time might play more important roles in regulation. One might expect that simply using a more demanding definition of conservation would work as well. However, Loots *et al.*[11] already adopted a stringent definition of conservation, requiring an ungapped alignment of at least 100 bp and at least 70% identity. The CNSs are obvious on a percent identity plot, such as shown in Fig. 2a. Note that CNS-1 is longer and is more similar between mouse and human than are the coding exons for the flanking interleukin genes. Looking in more distantly related species was not successful in this case, as the CNSs at 5q31 were not clearly detectable in chicken or *Fugu*, at least by a PCR assay[11]. Of course, determining the sequences of orthologous loci in chicken and fish and aligning those sequences with the mammalian loci would be a more sensitive (and expensive) way to address this issue.

This result contrasts with earlier work on enhancers of *HOX* genes, which are conserved between mammals and the teleost fish *Fugu rubripes*[14]. The ability to detect CNSs over this wider phylogenetic distance seems to result from the very slow rates of divergence of the *HOX* gene clusters. Kim *et al.*[15] found striking examples of CNSs throughout the *HOXA* cluster when the human sequence was compared to that of the primitive horn shark, *Heterodontus francisci*. Figure 2b shows examples of these in the region containing *HOXA10*, *HOXA9* and *HOXA7*. The human *HOXA* sequence is also compared with the mouse and *Fugu* sequences. The human and mouse sequences align throughout this segment. The *HOX* gene clusters, like the locus encoding T-cell receptor genes[16], is under intense selective pressure, and selection is exerted throughout the region. Thus the criteria used to

FIGURE 2. Percent identity plots comparing human and other sequences at three loci



The percent identity plot (pip) is a compact display of the results of aligning the human sequence with the designated second sequence. Each plot shows the positions in the human sequence and the percent identity of each aligning segment between gaps in the alignment. Along the top line of each plot, exons are indicated as black boxes and untranslated regions as light gray boxes. Within each plot, coding exons are underlayed with blue, and untranslated regions and introns are underlayed with yellow. (a) Comparison of part of the human 5q31 sequence (GenBank accession number AC004039.1) with the orthologous mouse sequence (AC005742.1). The conserved noncoding sequence (CNS) region of the pip is underlayed with red, and noncoding regions that align but do not fit the criteria in Loots *et al.*[11] (an ungapped alignment of at least 100 bp and at least 70% identity) are green. (b) Comparison of part of the human *HOXA* cluster (AC004080.1) with the orthologous sequence in mouse (AC015583.6) in the top panel, *Heterodontus francisci* (horn shark, AF224262.1) in the middle panel, and *Fugu rubripes* (teleost fish, FRU92573) in the bottom panel. Within the pips, CNSs found in comparison with *Heterodontus* are underlayed with red. (c) Comparison of part of the human *HBB* locus (U01317.1) with the mouse *Hbb* locus (X14061.1). Colors in the pip are orange for DNase hypersensitive sites (also indicated as open boxes along the top of the plot), red for a match that fits the criteria of Loots *et al.*[11] for a CNS, and green for other noncoding, aligning regions, many of which have been implicated in regulation. The pips were generated by PipMaker (http://bio.cse.psu.edu)[21].

identify CNSs at 5q31 finds many more CNSs in a human–mouse comparison of *HOX* genes than can be reasonably studied. However, extending the phylogenetic distance covered to that between mammals and fish (shark or pufferfish) reveals noncoding sequences that are under stronger selection, and hence likely to be involved in more critical functions. Of course, the noncoding sequences conserved between human and mouse, but not between human and fish, might indeed be functional in mammals, but an experimentalist would want to test the most highly conserved regions first.

On the other hand, criteria that work well for finding a CNS at one locus might be too stringent at another locus. Figure 2c shows a pip of the human and mouse sequences in the beta-globin locus control region and the promoter for the epsilon-globin gene, *HBE1*. Sequences at the DNase hypersensitive sites, and some sequences between them, are conserved in mammals and are needed for function of the locus control region (reviewed in Ref. 17). However, only one segment (located in HS2) fulfils the criteria used to define CNSs at 5q31. Thus a less stringent definition, or use of species phylogenetically closer to human, is appropriate at this locus. For other loci, a non-mammalian, warm-blooded vertebrate might be most informative. For example, Gottgens *et al.*[13] found that some noncoding sequences of the *SCL/TAL1* gene are strongly conserved between human and chicken, and they showed that one such CNS is a neural enhancer.

Thus the choice of appropriate species to compare in an effort to find CNSs that are the best candidates for function will depend on the locus being investigated. It is clear from many comparisons[6,8,9] that mouse and human sequences will be highly informative at many, and perhaps most, loci. This is good news, as the genomic sequence of both will be determined in the near future. Indeed, even shotgun sample sequencing of mouse is likely to reveal

Box 1. Glossary

Coding sequences
DNA sequences that code for proteins or many RNAs (rRNA, snRNA, tRNA).

Conserved noncoding sequences (CNSs)
In general, these are noncoding sequences that match in alignments of sequences between two species. One can define criteria that may be more likely to identify sequences under selective pressure, such as requiring a length of at least 100 bp and a percent identity of at least 70%[1].

Contig
A contiguous, or uninterrupted, DNA sequence generated from a set of overlapping sequences of shorter length.

Finished genomic DNA sequence
A contiguous sequence with <1 error per 10 000 bases.

Noncoding sequences
In general, these are DNA sequences that do not code for proteins or stable RNAs, such as the sequences between genes. However, the noncoding regions of protein-coding genes include the untranslated regions (even though these are present in mature mRNA) and introns (even though these are transcribed).

Percent identity plot (pip)
A compact display of the results of aligning two sequences, where the positions (in the first sequence) and percent identity of gap-free aligning segments are plotted, along with icons for features in the first sequence.

Working draft of the human genome
A set of DNA sequences representing coverage of about 90% of the human genome. The remaining 10% cannot be sequenced with current technology; much of it is highly repetitive DNA, such as that from centromeres. In the current public sequencing effort, mapped clones are initially 'shotgun' sequenced in an automated and efficient process. These shotgun data are assembled into a 'draft sequence' that covers most of the region of interest but still contains gaps and ambiguities. The second phase of sequencing fills the gaps and resolves ambiguities, producing a finished sequence. The working draft of the human genome is a combination of finished and draft sequence. The draft sequence has an average contig length of 15 kb, and about one error per 5000 bases.

Reference

1 Loots, G.G. *et al.* (2000) Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. *Science* 288, 136–140

much information about coding and noncoding sequences[18]. As these genomes are completed, however, the choice of additional species to sequence will be critical[19]. We hope that we will soon have a better idea of which portions of the mammalian genome are changing as slowly as the *HOX* clusters or more rapidly than *HBB*.

Software is available for finding conserved sequences

Finding conserved sequences requires both genomic DNA sequences and good software for aligning them. Both of these are now freely available to any investigator, so anyone can incorporate these methods into their research. The human genome sequences for many loci are either now or soon will be in the public domain, and the sequencing of mouse is under way[20]. Several alignment programs are available, many of them from public servers. The PipMaker server (http://bio.cse.psu.edu/) specializes in the alignment of extremely long genomic DNA sequences (up to two million base pairs in each sequence) and returns the results in compact, flexible and easily understood formats[21]. An additional server for comparative genomic sequence analysis, called VISTA (http://www-gsd.lbl.gov/vista/), is now available[22]. Use of such servers, in conjunction with the expanding array of publicly available resources, should make genomic analysis accessible to all interested investigators. The successes reported by Loots *et al.*[11] and others emphasize the benefit of incorporating rigorous analysis of genomic DNA sequences, including interspecies comparisons, at an early stage of projects in functional genomics.

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