Comparative studies of gene expression and the evolution of gene regulation

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Abstract | The hypothesis that differences in gene regulation have an important role in speciation and adaptation is more than 40 years old. With the advent of new sequencing technologies, we are able to characterize and study gene expression levels and associated regulatory mechanisms in a large number of individuals and species at an unprecedented resolution and scale. We have thus gained new insights into the evolutionary pressures that shape gene expression levels and have developed an appreciation for the relative importance of evolutionary changes in different regulatory genetic and epigenetic mechanisms. The current challenge is to link gene regulatory changes to adaptive evolution of complex phenotypes. Here we mainly focus on comparative studies in primates and how they are complemented by studies in model organisms.

RNA sequencing
(RNA-seq). An experimental protocol that uses next-generation sequencing technologies to sequence the RNA molecules within a biological sample in an effort to determine the primary sequence and relative abundance of each RNA type.

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A major objective of evolutionary genetics is to provide a mechanistic account of the genetic basis for inter-species phenotypic variation. The goal is to identify the genetic changes and molecular mechanisms that underlie phenotypic diversity, as well as to understand the evolutionary pressures under which phenotypic diversity evolves. Although the relative contribution of changes in gene regulation to adaptation continues to be debated[1,2], it has become clear that variation in gene expression patterns often plays a key part in the evolution of morphological phenotypes[3] as well as in a subset of other complex traits[4,5].

The notion that changes in gene regulation often cause phenotypic diversity is not new. More than four decades ago, Britten and Davidson hypothesized in a series of papers[6,7] that intergenic genomic regions (thought of by many at the time as 'junk DNA') have an important role in determining differences in gene regulatory patterns and, consequently, in phenotypic diversity. In 1975, King and Wilson[8] famously argued that the vast phenotypic differences between humans and chimpanzees are not likely to be explained solely by changes to structural proteins. They proposed that differences in gene regulation are likely to contribute to phenotypic differences between closely related species.

For nearly 30 years, however, these hypotheses could not be rigorously tested or challenged, mainly because relevant data on gene regulation could not be collected at an appropriate scale or resolution and because of difficulties in identifying regulatory elements in the genome. It was also unclear to what extent the environment affects gene expression phenotypes and whether it would at all be possible to detect genetic contributions to variation in gene regulation within or between species.

The past decade has seen tremendous developments in genomic technologies, which finally allowed investigators to apply high-throughput approaches to the study of gene expression patterns and associated regulatory mechanisms. For example, microarrays and now RNA sequencing (RNA-seq) enable genome-wide assessment of gene expression levels, and chromatin immunoprecipitation followed by sequencing (ChIP–seq) allows exploration of different aspects of regulatory mechanisms, such as transcription factor binding or histone modification. These advances provide the means to tackle outstanding questions regarding the evolution of gene regulation, including the characterization of the evolutionary forces that shape gene expression levels and the extent to which changes in different genetic and epigenetic mechanisms underlie regulatory variation. The relative importance of changes in gene regulation to phenotypic diversity and adaptation can now be studied with greater ease using these new techniques, although as we discuss below, a satisfying answer to this question still eludes us.

Expression quantitative trait loci
(eQTLs). Loci at which genetic allelic variation is associated with variation in gene expression levels.

Stabilizing selection
Natural selection against individuals that deviate from an intermediate optimum; this process tends to stabilize the phenotype. By contrast, directional selection pushes it towards either extreme.

This Review is focused on findings that emerge from comparative studies of gene regulation using cutting-edge genomic techniques. Studies that focus on variation in gene expression levels within species are discussed only briefly in this Review. It is important to note, however, that the body of work focused on within-species patterns has provided an important foundation for comparative studies by providing evidence that much of the observed variation in gene expression levels among individuals is heritable and can often be explained by corresponding genetic variation. Indeed it can often be mapped to specific loci referred to as expression quantitative trait loci (eQTLs)[9,10]. This finding provided a strong motivation for comparative studies to focus on expression levels as an important intermediate molecular phenotype: one that ultimately determines heritable variation in complex morphological and physiological phenotypes, including traits that evolved under natural selection.

Early large-scale comparative studies of gene expression levels have been previously reviewed[11,12]. Here, we discuss recent progress in comparative studies of gene expression and regulation, which are primarily based on the use of new sequencing technologies. We start with an overview of comparative studies of gene expression levels and then explore observations — focusing on primates — that shed light on the evolution of gene regulation and the associated genetic and epigenetic regulatory mechanisms. We discuss the connection between variation in gene regulation and variation in complex phenotypes and, in that context, point out important principal differences between comparative studies in primates and in model organisms. Finally, we comment on the possibilities to develop model systems that will allow us to study further the evolution of gene regulation in primates using experimental rather than strictly descriptive approaches.

Box 1 | **Comparative analysis of RNA sequencing data**

Comparative studies of gene expression levels using RNA sequencing (RNA-seq) overcome many of the traditional limitations that are associated with microarray data, but they are not free of challenges. Most challenges are common to all RNA-seq studies and relate to the count nature of the RNA-seq data, the need to normalize and standardize the data and the desire to account for confounding and biasing factors (such as differences in transcript length or GC content across genes). One challenge, however, is fairly specific to comparative studies: the requirement of defining the transcriptome. This is necessary because comparisons of expression level estimates can only be interpreted in the context of defined transcriptional units (for example, comparison of the expression levels of exons, specific transcripts or genes). When RNA is being sequenced from a species for which a well-annotated genome is available, RNA-seq reads can be aligned to the previously defined transcriptome, and expression levels can be estimated on the basis of the number of aligned reads. The problem is that only a few genomes are well annotated.

When a genome is available but not well annotated, two approaches can be used to define transcriptional units. The first approach relies on the functional annotations from a closely related genome, and this approach has to overcome the challenge of accurately defining orthology. A conservative definition of orthology — namely, requiring high sequence similarity for assignments — risks excluding a large fraction of transcriptional units from the analysis, whereas relaxed criteria (that is, accepting weaker evidence for homology) can result in erroneous orthology assignments. The second approach is to align RNA-seq reads to the genome sequence and *de novo* to define expressed transcriptional units. This task is far from trivial, as it requires distinguishing foreground expression levels from the background (such as sequencing reads that correspond to unspliced introns).

When a genome sequence is not available, *de novo* transcriptome assembly is required. This is a particularly challenging task, because it does not rely on an alignment of the sequencing reads to a known genome. Despite this technical challenge, for the purpose of comparing expression levels across species, the data obtained by *de novo* transcriptome assembly are expected to have the same properties as those obtained from defining transcriptional units on the basis of aligning RNA-seq reads to a genome. Thus, transcriptome assembly is an attractive approach for studies on any species for which genome sequences are not yet available. That said, with the rapid decrease in sequencing costs and the corresponding increase in sequencing capacity, it might be reasonable to expect that sequencing large (for example, mammalian) genomes may not be a prohibitive enterprise in the near future.

Comparative studies of gene expression

A common approach towards the study of the evolution of gene regulation is to characterize and compare gene expression levels across species with the goal of understanding genetically regulated inter-species differences. Before the advent of next-generation sequencing technologies, the only practical approach towards measuring and comparing gene expression levels on a genome-wide scale was to use DNA microarrays. Comparative studies using arrays have resulted in important insight into the evolution of gene regulation (reviewed in REFS 11–13). Yet, microarrays can only be designed for species with available sequenced genomes. By contrast, using RNA-seq techniques, it is possible to measure and to compare gene expression levels across practically any combination of species[14,15], even when genomic sequences are not yet available[16]. In addition, RNA-seq data allow estimation of gene expression levels at a much broader dynamic range than microarrays, identify previously unannotated transcripts, compare alternative splicing patterns and exon usage across species[17] and characterize genetic diversity in expressed genes[16]. Although comparative analysis of RNA-seq data is challenging and remains an area of active research (BOX 1), the advantages of this methodology over microarrays are clear[14,18].

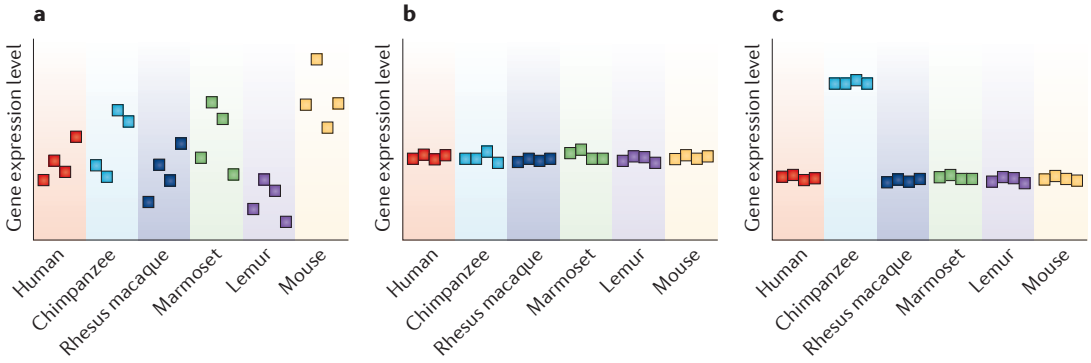
Action of natural selection on gene regulation

One approach towards studying the evolutionary forces that shape gene regulation is to identify gene expression patterns that can be explained by different evolutionary scenarios, such as stabilizing selection or directional selection on gene regulation. To do so, it is necessary to distinguish between the environmental and genetic effects on gene regulation as well as to control for a large number of potential sources of variation and error. These can be technical sources, such as variation in sample quality and batch effects (for example, owing to differences in collection protocols), or biological sources, such as variation due to sex, age and circadian rhythm. In addition, physiological, morphological and environmental differences between species (for example, differences in diets) are also expected to contribute to differences in gene expression levels across species.

Studies in model organisms typically match the environmental conditions across individuals and take measures to minimize or to control the technical and biological variation associated with the experiment.

Ranking-based approach
Genome-wide studies often use model-free ranking to prioritize candidate genes. Ranking is performed on the basis of properties that are expected to be informative with respect to the desired trait (for example, nucleotide diversity across populations when the desired traits is evidence for natural selection).

Box 2 | **The signatures of natural selection on gene regulation**



How is it possible to distinguish between different modes of gene expression evolution? One approach is to look for departures from a null model of a given evolutionary scenario. At the sequence level, the most commonly used null is the neutral model, which proposes that some alleles are strongly deleterious, are subjected to strong purifying selection and thus are never seen in a sample, whereas the alleles that do segregate in the population are selectively neutral[106,107]. In the case of a quantitative phenotype, such as gene expression levels, evolutionary constraint is likely to take the form of stabilizing selection, which maintains a constant mean and reduces the variance of the trait[108,109]. However, as discussed in the main text, it is difficult to specify the expectations under the null model for non-model species. An alternative is to use an empirical approach to identify gene expression patterns that are likely to have evolved under natural selection.

For example, if gene regulation evolves under stabilizing selection, genes are expected to show little variation in expression levels within and between species. By contrast, under directional selection in a particular lineage, genes are expected to show a substantial shift in the mean expression level in that one lineage and to show little variation in expression levels among individuals within a species[22]. This is illustrated schematically in the figure: gene expression levels (*y*-axis) are plotted for four individuals from each of six mammalian species. In panel **a**, variation in gene expression level is high both within and between species. This might not be unexpected, given that it is difficult to stage tissues and to minimize environmental effects on gene regulation in a comparative study. In panel **b**, little variation in gene expression levels is observed both within and between species. The most likely explanation for such a pattern, especially in the face of the technical limitations that are associated with comparative studies using non-model organisms, is that gene regulation evolves under stabilizing selection. The pattern shown in panel **c** indicates a change in gene expression level in the chimpanzee lineage, which is consistent with directional selection on gene regulation in chimpanzee. However, alternative explanations — such as lineage-specific relaxation of evolutionary constraint or lineage-specific difference in environment — are difficult to exclude.

The inference of selection based on the empirical approach relies on the ranking of expression level variation within and between species, not on direct evidence for the presence or absence of natural selection. Although statistical analyses are typically used to rank genes on the basis of their gene expression patterns, this ranking-based approach should be considered heuristic and model-free. It is difficult to apply less heuristic approaches to the comparative analysis of gene expression levels in primates because it is not possible directly to study the mutational input for gene expression variation in these species, nor is it possible to establish experimentally what levels of gene expression divergence indicate the action of natural selection rather than low mutational input.

Similar empirical approaches are used in other types of genome-wide data analyses: for example, in scanning sequence data for evidence of recent natural selection on specific genes[110–113]. The general rationale is that genomic regions or genes ranked at the top of the list have nucleotide diversity or expression patterns that provide the most compelling evidence for the action of natural selection. It is therefore expected that genes at the top of the list would be enriched for true targets of recent natural selection. It is recognized, however, that not all genomic regions at the top of list (regardless of the cutoff chosen) are indeed targets of natural selection and, conversely, not all true targets of natural selection will be at the top of the list[114,115].

Comparative studies in model species can obtain evidence for natural selection on quantitative traits (such as gene expression levels) by testing for deviations from specified null models[19–21] (BOX 2). Broadly speaking, this approach requires estimates of the expected interspecies variation in gene expression levels under the null (for instance, under a model of no selection), deviation from which is interpreted as evidence for alternative scenarios (for example, evidence for the action of natural selection). Such an approach relies on a number of parameter estimates (for example, the mutation accumulation rate), which need to be estimated or measured independently[13].

In non-model organisms, notably in primates, it is often impossible or impractical to estimate directly the parameters of a null model of the evolution of gene expression. One alternative to specifying an explicit model is to take an empirical approach, in which genes are first ranked according to their patterns of expression levels within and between species and are then evaluated for fit to expectations under different evolutionary scenarios (BOX 2). The goal of the empirical approach is to identify specific patterns of heritable gene expression levels that are consistent with the action of natural selection. However, in non-model organisms it is often impossible to distinguish between

technical and biological variance or to match the environment across individuals of different species. As a result, some observations from comparative studies of gene regulation in such species should be interpreted with caution.

The observation of inter-species differences in gene expression levels is inherently difficult to interpret, because environmental and genetic explanations can be completely confounded. It is reasonable to assume that differences in the environment experienced by different individuals and species will generally result in perturbation of gene regulation and lead to an increase in variation of gene expression levels. By contrast, genes that have low variation in expression levels across individuals and species are probably those that are robust to environmental differences. It can therefore be concluded with considerable confidence that the regulation of genes with constant expression levels across individuals and species is genetically controlled. Low variation in gene expression levels across species is consistent with the action of stabilizing selection on gene regulation[22]. When a difference in gene expression is seen in a specific lineage (BOX 2) — for example, a higher expression level observed exclusively in humans — this may indicate the action of directional selection on gene regulation in that lineage. Alternatively, it may be a consequence of a specific environmental influence on that lineage (for example, the consumption of cooked food in the case of humans[23,24]).

Comparative studies in primates

Differences in gene regulation between humans and other primates may ultimately be used to explain the molecular basis for human-specific traits. For example, it was hypothesized that human-specific gene expression patterns in the brain[25,26] might underlie functional, developmental and perhaps cognitive differences between humans and other apes. A recent comparative study that incorporated temporal resolution into the study design found potential differences in the timing of gene expression in the brain across primates[27], which might be related to inter-species differences in the timing of developmental processes. Genes with potential roles in neural development showed a marked delay in expression timing in human brain samples compared with chimpanzees and rhesus macaques[27]. More generally, several major principles have emerged from comparative studies of gene expression among primates (and in some cases among other species as well).

Selective constraint. Although the notion that the expression levels of most genes are shaped by natural selection was previously debated[28], multiple studies now support the conclusion that the regulation of a large subset of genes and pathways evolve under natural selection in primates[27,29,30]. Comparative gene expression data in apes and old-world monkeys suggest that the regulation of a large subset of genes is evolving under selective constraint. Indeed, comparative studies[27,29,30] have found that the extent of inter-species variation in gene expression levels can often be explained by variation in gene expression within a species, which is consistent with the action of stabilizing selection on gene regulation. More generally, although there is much uncertainty about the relevant values of important parameters for a standard neutral model of gene expression evolution in primates (as discussed above and in BOX 2), even when conservative estimates are used for generation time and mutation rates, the overwhelming majority of genes exhibit far less between-species variation in gene expression levels than would be expected if all regulatory mutations were neutral[19]. These studies, however, had the minor weakness that they relied only on comparative data from closely related species (typically, humans, chimpanzees and rhesus macaques). Thus it remained possible that the inference of widespread selective constraint on gene regulation could be explained by the lack of mutations that effected gene expression owing to chance. That is, because regulatory elements constitute a small fraction of the genome, gene expression patterns among closely related species may appear to be under constraint if not enough time has passed since the most recent common ancestor for regulatory substitutions to accumulate in substantial numbers.

More recently, an RNA-seq study has looked at gene expression levels and genetic diversity in livers from 16 mammalian species, including humans and 11 non-human primates[16]. All liver samples for this study were collected post-mortem, and it was therefore not possible to stage the tissues or to control for possible environmental effects across species. Nevertheless, expression patterns of many genes showed remarkable conservation, suggesting a strong genetic component in their regulation as well as the action of stabilizing selection over hundreds of millions of years.

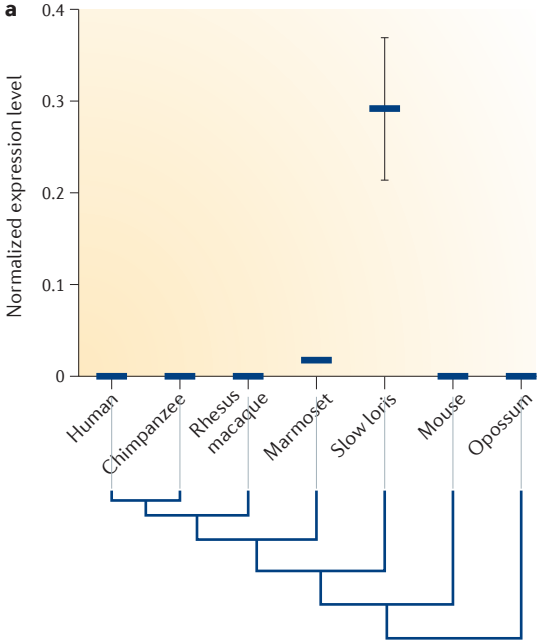
Directional selection. There is also evidence that the regulation of some genes — 10–30% of genes (depending on the tissue or cell type studied)[22,31,32] — has evolved under directional (positive) selection. For instance, the comparative RNA-seq study of 16 species[16] also identified lineage-specific changes in expression levels; an example is shown in BOX 3. However, as we discuss above, inferring positive directional selection on gene regulation in non-model species is more complicated than inferring selective constraint. Although a lineage-specific change in gene expression level may be consistent with the action of directional selection — that is, it is reasonable to assume that directional selection on gene regulation would result in inter-species differences in gene expression levels — it is unclear how many regulatory differences are truly the result of selection. Alternative explanations for gene expression differences between species, such as consistent inter-species differences in environments, are often difficult to exclude, especially in primates. By ranking genes according to inter-individual variation in expression levels, it is possible confidently to assume that the set of genes that are differentially expressed among species and that are associated with low within-species variance is, as a group, enriched for targets of selection compared to genes that are not differentially expressed between

Neutral model
A model stating that alleles that reach sufficient frequency within a population to be sampled, or that are fixed between species, are selectively neutral, whereas a subset of alleles are too strongly deleterious either to segregate within a population in appreciable frequencies or to reach fixation.

Vitamin A toxicity
Having too much vitamin A in the body. This can lead to multiple clinically abnormal conditions including decreased appetite, softening of the skull bone, nausea, vomiting, blurry vision, headaches and hair loss.

Box 3 | **Inter-species regulatory differences and ecological adaptation: a case study**

Comparative studies of gene expression levels might reveal the molecular signatures of ecological adaptations. An illustrative example of this is provided by the work of Perry and colleagues[16], who found that the expression levels of short chain dehydrogenase/reductase family 16C, member 5 (*SDR16C5*; panel **a** of the figure, *y*-axis) are elevated in the livers of marmosets and slow lorises compared with all other studied primates (in the livers of most other primates, the expression of this genes could not be detected). SDR16C5, an epidermal retinol dehydrogenase, is involved in the first, rate-limiting step of retinol (vitamin A) metabolism. Retinol is a derivative of isoprene, which is the monomer of latex. Slow lorises and marmosets feed extensively on tree exudates[116,117], which may include gums, saps and latex; a marmoset gouging tree bark is shown in panel **b** of the figure. Among the species considered in this study[16], only marmosets and slow lorises have apparent craniofacial adaptations for tree gouging. It is not known how exudates are digested in primates, but this process is thought to be aided by bacterial fermentation in the gut. In this case, there may be large quantities of the digestive products, such as retinol, absorbed through the large intestine, which may then be filtered by the liver. The intermediate-to-high expression levels of *SDR16C5* that are found exclusively in the liver tissues of slow lorises and marmosets could represent convergent adaptation against the fitness-reducing effects of vitamin A toxicity. Of course, such hypotheses based on single-gene observations should be considered to be highly tenuous. Nevertheless, this information may be valuable if it ultimately leads to further study and to a better understanding of diet-related adaptations and evolutionary ecology in primates. Data for panel **a** are taken from REF. 16. Image in panel **b** © Ana Karinne Lima.



species (BOX 2). Yet, it may always be difficult to identify with confidence the individual genes whose regulation evolved under positive selection.

Tissue specificity. Another question is whether gene regulation in primates evolves under tissue-specific selection pressures. A recent RNA-seq study[15] estimated gene expression levels in six different tissues from nine mammalian species (including humans and all four great apes) and showed substantially different rates of transcriptome evolution across tissues. This study[15] identified 145 gene expression network modules that had lineage-specific expression patterns, which may indicate the action of species-specific and tissue-specific directional selection on gene regulation. This study also found 33 organ-specific gene expression network modules that are conserved across these mammals and are enriched with genes involved in biological processes that are intuitively considered to be typical for each of the studied tissues (for example, synaptic transmission in the brain). Similar patterns were observed in a more limited comparative study in humans, chimpanzees and rhesus macaques that focused on gene expression measurements from hearts, livers and kidneys from multiple individuals[32]. In the most extreme cases, the observed inter-species expression patterns of a subset of genes were consistent with the action of stabilizing selection in one tissue (for example, liver) and the action of lineage-specific directional selection in another tissue (for example, heart). The results of these studies are consistent with the idea that adaptation may more commonly proceed through regulatory changes rather than structural (that is, coding) changes, because regulatory mutations have spatially or temporally circumscribed effects.

Alternative splicing. The third emerging principle is that inter-species differences in gene expression levels seem only rarely to be explained by differences in alternative splicing between species. This may seem surprising, because alternative splicing and changes in exon usage could provide an intuitive mechanism by which to introduce functional variation to structural proteins. Yet, only a few instances of inter-species differences in exon usage have been observed[15,16,30]. For example, a recent study sequenced liver RNA from males and

females of humans, chimpanzees and rhesus macaques and characterized gene and exon-specific expression levels. This study showed that although sexually dimorphic differences in exon usage are fairly common, sexually dimorphic gene expression levels and alternative splicing patterns are largely conserved between species[30]. A caveat of this result is that non-human primate transcriptomes are not well annotated, so that the probability of missing an exon expressed only in a non-human primate may be high. However, such technical explanations are unlikely to account for the observation that nearly all expressed exons in humans are also expressed in non-human primates. Given the sequencing depth of recent comparative studies, explanations based on a lack of power are unlikely either.

As can be seen, comparative studies in primates, although challenging, have resulted in important insights into the evolution of gene expression levels. Yet, we are also finding that gene expression patterns alone provide little insight into the adaptive phenotypes, molecular mechanisms or even the specific biological processes that are involved in the observed changes in gene expression levels. The question at this point is how to move beyond descriptive studies of gene expression levels across species.

From gene expression to regulatory mechanisms

There are two general approaches to 'move beyond' a simple description of the evolution of gene expression patterns. One approach is to perform functional experiments to understand adaptive phenotypes; the question that is typically being asked is 'what differences in phenotype do these changes in gene expression levels underlie?' The other general approach is to perform comparative studies of the underlying regulatory mechanisms: in effect pursuing the opposite direction, as it were, asking 'what changes in regulatory mechanisms explain the observed differences in gene expression levels?' The latter approach does not provide insight into phenotypes, but it addresses other outstanding questions regarding the mechanisms that shape regulatory evolution (FIG. 1). In this section, we discuss the progress that has been made using comparative studies of regulatory mechanisms.

A large number of gene regulatory mechanisms are reasonably well understood (for example, those that are involved in transcription initiation; reviewed in REF. 33). Yet, we still know little about the relative contribution of changes in different genetic and epigenetic regulatory mechanisms to the evolution of gene expression levels. From an evolutionary biologists' perspective, uncovering the mechanisms of regulatory adaptations will reveal what types of mutations underlie inter-species differences in gene expression levels and reveal the genetic loci that are likely to underlie phenotypic adaptation and speciation. From a biomedical perspective, understanding the mechanisms of regulatory evolution, especially in primates, is expected to help us to guide the search for functional elements in the human genome, which are likely disproportionally to harbour disease-causing mutations[34].

Comparative studies of regulatory mechanisms need to address the same challenges and difficulties that were discussed in the context of comparative gene expression studies. Genetic and epigenetic regulatory profiles are influenced by environment, cell composition and circadian rhythm, just to name a few potentially confounding effects. It is easier to control for these effects when conducting studies in model organisms but, nevertheless, important trends have emerged from comparative studies in primates as well.

Comparisons of transcription factor binding

In one of the first sequencing-based comparative functional genome-wide studies of transcription factor binding[35], ChIP–seq was performed for two hepatic transcription factors in liver samples from five vertebrates, including humans. The results showed that most binding locations are species-specific. Of the ~16,000–30,000 binding sites identified in each species, only 35 were shared across all five species, and only 344 were shared by the three mammalian species studied (namely, humans, mice and dogs). A study of RNA polymerase II binding[36] showed that 32% of binding locations in immortalized B cell lines differed between humans and chimpanzees (although it is important to note that they only had one chimpanzee sample), and 25% of sites differed between human individuals. These studies suggest that evolutionary turnover of transcription factor binding sites is rapid and that, on a genome-wide scale, most binding locations may not be conserved even across closely related species (FIG. 2). However, because these studies did not collect comparative gene expression data from the same samples, it was not possible to assess the degree to which differences in transcription factor binding might account for inter-species differences in gene expression levels. As a result, it cannot be excluded that those binding events that have effects on gene regulation are more conserved than suggested by general genome-wide patterns.

A different approach was taken in a study[37] that introduced a functional and freely segregating copy of human chromosome 21 into a mouse to generate a model of trisomy 21. Examination of the binding locations of three transcription factors — hepatocyte nuclear factor 1α (HNF1α), HNF4α and HNF6 — in livers from these mice and in human hepatocytes showed that 85–92% of binding locations on human chromosome 21 in the mouse coincided with binding sites observed in normal human hepatocytes[38]. Moreover, the expression profiles of genes on human chromosome 21 in mouse hepatocytes were highly correlated with those from human hepatocytes. Thus, in this case, differences in the cellular environment between human and mouse livers resulted in little change in transcription factor binding or gene expression patterns. The important inference from this study is that the sequence of human chromosome 21 appears to encode sufficient information to result in faithful regulatory output in mice: that is, regardless of the cellular environment.

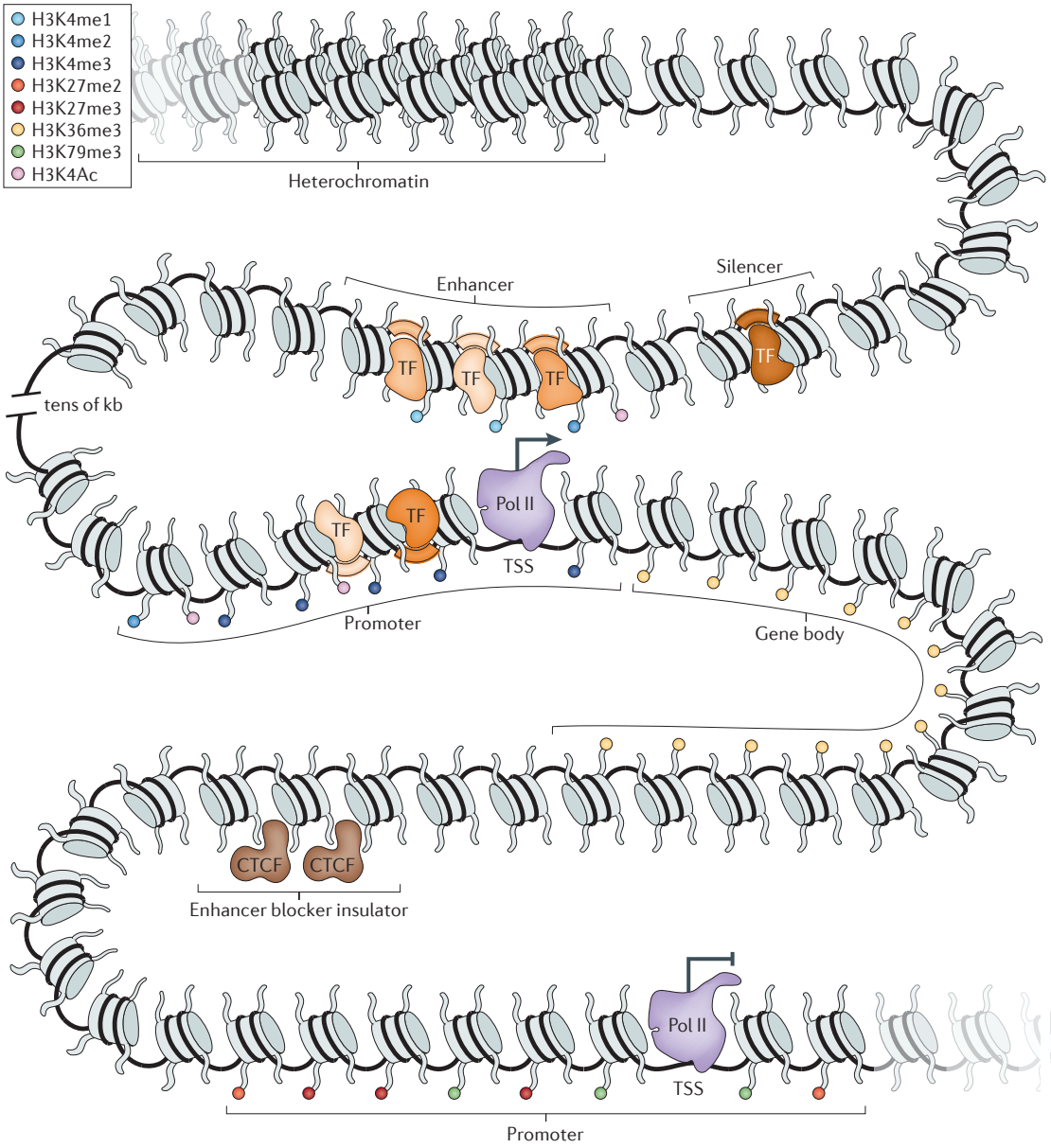


Figure 1 | **Regulatory mechanisms that can be investigated using comparative genomic approaches.** Changes in a large number of genetic and epigenetic regulatory mechanisms can underlie inter-species differences in gene expression levels. Next-generation sequencing technologies allow us to obtain genome-wide profiles of transcription factor binding and epigenetic markers and thus to identify correlations between variation in gene expression and variation in regulatory mechanisms. Using this paradigm, current studies are actively estimating the relative contribution of changes in different mechanisms to regulatory evolution, including chromatin accessibility (using DNaseI sequencing), nucleosome positions (using MNase sequencing), transcription factor binding (using chromatin immunoprecipitation followed by sequencing (ChIP–seq)), promoter methylation profiling (using microarrays or bisulphite sequencing) and a number of histone modification profiles (using ChIP–seq). H3K4me1, monomethylation of histone H3 at lysine 4; Pol II, RNA polymerase II; TF, transcription factor; TSS, transcription start site. The figure is modified, with permission, from REF. 118 © (2010) Wiley.

MNase sequencing
Sequencing of chromatin that has been treated with micrococcal nuclease (MNase), which preferentially cuts linker DNA connecting two nucleosomes. MNase sequencing can be used to map nucleosome positions.

Comparisons of epigenetic profiles

Another trend that emerges from comparative studies of regulatory mechanisms, especially in primates, is that a substantial fraction of gene expression differences across species can be explained by inter-species changes in epigenetic mechanisms. For instance, genomic regions that are associated with trimethylation of histone H3 at lysine 4 (H3K4me3) — a histone mark that denotes active transcription[39] — were characterized using ChIP–seq in immortalized B cells from humans, chimpanzees and rhesus macaques[40], and RNA-seq data were also collected from the same samples. Overall, there were large differences in the patterns of this histone modification across the three

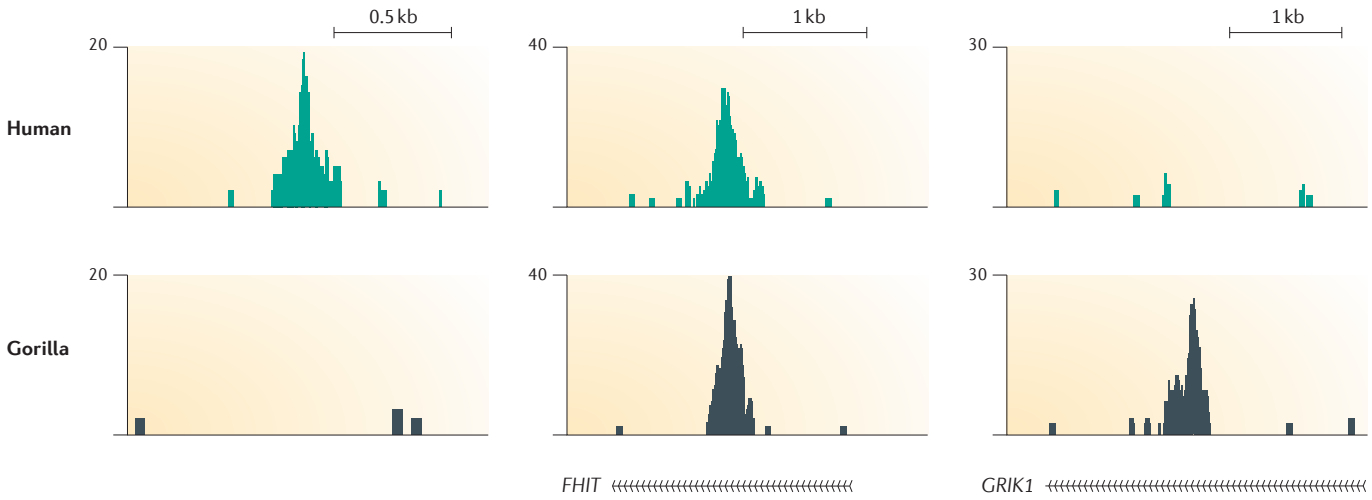


Figure 2 | **Inter-species differences in transcription factor binding.** Ward, Odom and colleagues[119] performed and analysed comparative chromatin immunoprecipitation followed by sequencing (ChIP–seq) experiments for the transcriptional regulator CTCF in human and gorilla cell lines[119]. After ChIP–seq reads are mapped to the respective genomes, the resulting peaks (read counts are plotted on the *y*-axis) indicate the locations of chromatin enrichment and hence of CTCF binding. Examples are shown of a site bound in humans but not in gorillas within 2 kb of the G-protein-coupled receptor 88 (*GPR88*) gene (this gene is not shown in the figure), a shared site at fragile histidine triad (*FHIT*) and a site bound in gorillas but not in humans at glutamate receptor, ionotropic, kainate 1 (*GRIK1*). The data for this figure are taken from REF. 122.

species, but a high degree of conservation near transcription start sites (TSSs), where H3K4me3 is most likely to be functional. The subset of genes associated with inter-species differences in H3K4me3 modification near their TSS were also more likely to be differentially expressed between species. Because this study looked at correlations between gene expression data and H3K4me3 ChIP–seq data, direct causal inference was impossible. Nevertheless, on the basis of previous work on regulation by histone modifications[41,42], the authors estimated that up to 7% of gene expression differences across the three species could be accounted for by changes in H3K4me3 status.

A similar approach was used to study correlations between gene expression levels and promoter DNA methylation status in livers, hearts and kidneys from humans and chimpanzees[43]. As expected, variation in methylation states between different tissues was greater than between species. Moreover, tissue-specific promoter methylation profiles were generally conserved. This result is consistent with other studies that reported a large overlap in methylation profiles across primates — for example, in human and chimpanzee sperm[44], or in human, chimpanzee and orangutan neutrophils[45]. That said, differentially expressed genes between humans and chimpanzees were often associated with promoter methylation differences, regardless of tissue type. In light of a large body of work that supports the causal effects of promoter DNA methylation on gene regulation[46,47], the authors estimated that as much as 12–18% (depending on the tissue) of inter-species differences in gene expression levels could be explained by changes in promoter methylation profiles.

As these examples show, most comparative work conducted to date has focused on mechanisms of transcriptional initiation. A few studies, however, are looking elsewhere for factors that can influence gene regulation during evolution. For instance, changes in microRNA expression levels, which are expected to affect rates of mRNA decay, could account for ~2–4% of gene expression differences across the prefrontal cortex of humans, chimpanzees and rhesus macaques[48,49].

From gene expression to complex phenotypes

Comparative studies of regulatory mechanisms in primates rely on correlations between different measurements. Despite important insights, without direct experimentation it is difficult to assess causality or the impact of changes in regulatory mechanisms on gene expression levels at the organism level. Functional experimentation in humans and other apes is technically limited to a few immortalized cell lines, non-invasively sampled tissues or post-mortem samples, which are difficult to stage. In most cases, it is difficult to infer which phenotypic adaptation was mediated by species-specific changes in gene expression levels or even how to formulate specific hypotheses for further experiments. Even when the mechanism and specific regulatory sequence elements underlying the expression change may be known (for example, using the approaches described above to characterize the regulatory mechanisms), the phenotypes that are being affected by the regulatory change are typically unknown. Because of the obvious ethical and practical limitations on experimentation in primates (especially apes), it is difficult to envision an approach that will allow follow-up of these observations and testing of their functional relevance. To circumvent

these limitations, several studies have used model organisms to address specific hypotheses inspired by comparative analysis of gene regulation in primates.

For example, McLean and colleagues[50] investigated the phenotype associated with a human-specific 5 kb deletion upstream of the androgen receptor gene, which includes sequence that is conserved in other mammals (and therefore is likely to be functional). Constructs containing the mouse and chimpanzee versions of this region directed reporter gene expression in the facial vibrissae and genital tubercle of transgenic mice. Because the androgen receptor gene is implicated in the development of sensory vibrissae and penile spines[51,52], the loss of this tissue-specific enhancer in the human lineage was interpreted as being a causal mechanism for the human-specific loss of these morphological properties.

Other studies, using similar approaches that involve functional experimentation in model systems, identified an ancient enhancer that may have recently gained a human-specific function linked with the evolution of the human thumb[42], a change in non-coding RNA sequence that may be linked to cortical development[53] and a human-specific change in the forkhead transcription factor *FOXP2*, which might be related to the development of language[54,55]. It should be noted, however, that in most of these studies, model organisms are used to recapitulate gene regulatory differences between primates and to study them with high spatial and temporal resolution[56]. Therefore, the inference about function requires two important assumptions to be made. First, the effects of gene regulatory changes on complex phenotypes are identical in model organisms and in primates, including humans. This assumption may be difficult to accept in some cases: for example, when the phenotype under consideration is language. The second assumption is that no other regulatory changes could manifest in similar patterns. For example, if multiple enhancers drive nearly identical spatiotemporal expression patterns of a reporter gene, it is unclear how to identify the particular enhancer whose evolution may be associated with a derived trait. At the moment, data are not yet available to estimate how often this assumption is reasonable.

Comparative studies in model organisms

Because a broad range of experimental manipulations are possible in model organisms, studies that focus on model species can move beyond simple comparisons of gene expression and offer deep insights into the causal relationship between regulatory changes and phenotypic evolution. Consider, for example, a pair of species that are distinguished by a specific difference in morphology, physiology or behaviour. Such a difference might result in a fitness benefit in the environments that the species inhabit, thereby revealing a selective pressure under which it has evolved (demonstrating this is often quite challenging; see REF. 57 for a recent Review). The two species may be sufficiently closely related to permit crosses, in which the genetic determinants of the interspecies phenotypic differences could be mapped. It is then possible to use different techniques (for example, positional cloning) to identify the specific mutations and molecular mechanisms that underlie the phenotypic divergence and provide evidence for causality.

A compelling example is the case of pelvic fin reduction in a threespine stickleback (*Gasterosteus aculeatus*). Repeated instances of pelvic reduction are thought to be adaptive and associated with invasions into freshwater habitats. Paired-like homeodomain transcription factor 1 (*Pitx1*), a gene encoding a transcription factor involved in pelvic fin development, has been identified as a candidate locus that is responsible for this morphological change[58]. Fine mapping[59] pointed to a putative regulatory element upstream of *Pitx1* as the causal locus. The deletion of this regulatory element, which population genetic data suggest has been subjected to positive selection, was hypothesized to result in a difference in *Pitx1* expression pattern and, ultimately, in a reduced pelvic fin. This hypothesis was supported by transgenic experiments that demonstrated that the candidate non-coding region is indeed a regulatory enhancer. Furthermore, the reduced-pelvic phenotype could be reversed by using a transgene containing the candidate genomic region. Similarly compelling examples are the change in a *cis*-regulation of the *agouti* gene during the evolution of camouflage coloration in *Peromyscus* mice[60] and the regulatory change of the *optix* gene, which has been identified as the site of repeated evolution of the wing colour patterns responsible for mimicry in *Heliconius* butterflies[61].

More generally, work in model species suggests that divergence of gene expression levels of individual loci may be subtle[62,63], but that even small changes in the regulatory state can cause substantial phenotypic divergence[64] associated with fitness effects[65,66]. This view emphasizes the complex polygenic nature of the evolution of gene expression[67], one in which epistatic interactions[68,69] and interactions with the environment[70] are important. That said, studies in which both the evolutionary history and the molecular mechanisms are well understood remain rare. By contrast, a few studies in model organisms have identified clear connections between changes in gene regulation and differences in phenotypes, which are assumed to be adaptive. Although the plausible scenario of adaptation can often be proposed, the exact nature of it remains elusive. Examples from plants, fungi and animals demonstrate the breadth of this phenomenon (for example, see REFS 68,71–74). Of course, evolution of many traits is not caused by changes in gene regulation[2,3]. Dramatic examples include a single amino acid mutation in the melanocortin-1 receptor gene causing pigmentation differences in beach mice[75] and aquaporin gene loss in natural populations of *Saccharomyces cerevisiae*[76].

Observations from studies of model organisms

In contrast to studies in primates, studies in model organisms have resulted in much more direct insight into the mechanisms underlying the evolution of gene regulation. For instance, it is difficult to obtain data that conclusively support regulatory changes in *cis* or *trans*

Enhancer
A region of DNA that binds to proteins whose function is to promote transcription of genes.

Positional cloning
A method for identifying the location of a risk variant within a candidate region. Overlapping clones covering the candidate region are typed, and segments that co-segregate perfectly with the disease are identified. These clones are the most likely location of the risk variant.

Pelvic fin
The fins that are attached to the pelvic girdle on the lower surface of the fish body. They help to control the direction of movement.

Mimicry
When an organism benefits from copying the phenotype of another organism.

Trans-regulatory elements
Regulatory elements that can affect the transcription rates of both alleles of a gene (examples include transcription factors and small regulatory RNAs). By contrast, *cis*-regulatory elements have an allele-specific regulatory effect.

Transposable elements
DNA sequences that can change their position in the genome.

Induced pluripotent stem cells
(iPSCs). These are derived from somatic cells by 'reprogramming' or de-differentiation triggered by the transfection of pluripotency genes, which alters the somatic cells to a state that is similar to that of embryonic stem cells.

in primates (the regulatory inferences discussed above cannot easily be validated or confirmed), but this has been done many times in model systems. Changes in *cis* elements appear to be more commonly responsible for inter-species differences in gene expression patterns than changes in *trans*, as shown in yeast and flies[77–80]. One mechanism that can lead to *cis*-regulatory divergence is a rapid turnover of transcription factor binding sites[81], which in turn could cause different transcription factor binding profiles, even between closely related species[82]. Changes in *trans*-regulatory elements (such as transcription factors and regulatory RNAs) have also been documented in yeast[79,83], and there is considerable evidence of co-evolution of *cis* and *trans* regulatory elements in various species[79,84,85].

In addition, several lines of evidence implicate chromatin state as an important player in the evolution of gene expression. Circumstantial evidence for the importance of this mechanism comes from studies in primates as well[86,87], but in model systems it is possible to demonstrate causality directly. Studies in yeast have shown that despite an overall similarity in nucleosome-positioning profiles, genes with divergent expression often show divergent chromatin organization[88–91]. Furthermore, certain properties of nucleotide sequences predispose promoters to evolve divergent gene expression more readily, perhaps through changes in chromatin structure[92]. For example, deletions of chromatin factors in yeast revealed previously cryptic gene expression differences, suggesting that these proteins buffer regulatory variation[93].

Recent experimental results in model systems[73,94–96] are also resurrecting the classical idea that transposable elements, containing pre-existing transcription factor binding sites, could insert in the vicinity of regulatory loci and could serve as a source of novel regulatory elements[6]. It appears that latent regulatory activity can be located in introns[74] and even in deteriorating coding sequences[97]. Whereas most studies discussed here considered transcriptional gene regulation, many other molecular processes regulate gene expression and can thus contribute to evolution of gene expression and phenotypes[98,99].

Conclusions

Genomic technologies allow us to characterize variation in gene expression levels within and between species with relative ease. As might be expected, the data suggest that the regulation of most genes evolved under evolutionary constraint, although subsets of genes whose regulation is likely to have evolved under directional selection can also be found. The challenge is to move beyond comparative descriptions of gene expression levels to the study of the underlying mechanisms and the connection between regulatory evolution and ultimate adaptation of complex phenotypes.

The lofty promise of genomics — to predict functional elements, including regulatory loci, on the basis of primary sequence — is becoming a reality. Major advances have been made in developing quantitative predictions of gene expression patterns on the basis of *cis*-regulatory sequences[100]. At first, these models primarily considered interactions of transcription factors with DNA[101,102], but more recently they have started to incorporate nucleosome-positioning information[103,104], making predictions more accurate and biologically realistic. Much work is still required, but as more sophisticated models are developed, we are likely to improve on our current ability to predict gene expression patterns from the sequences of their regulatory elements[105]. This, in turn, will help to determine which of the millions of nucleotide differences between the genomes of related species are responsible for their divergent patterns of gene regulation.

Functional studies of variation in complex phenotypes, however, will always be needed to validate model predictions, and these must involve empirical approaches. As we have discussed, although progress has been slow in all systems, effective experiments can be designed for model organisms. It is possible to reveal the causal relationships between differences in gene expression levels, the underlying regulatory mechanisms and the evolution of complex phenotypes. In primates, the only functional approach available thus far is to rely on experimentation on model systems, which is a useful approach at times, but the results are often somewhat difficult to interpret. If we are ever to use comparative functional approaches to study the genetic architecture that underlies regulatory adaptation and its phenotypic consequences in humans and other apes, a new paradigm is needed. Perhaps the advent of induced pluripotent stem cells (iPSCs) will provide an alternative system for functional studies in primates. iPSCs can be differentiated into various cell types and can thus provide a surrogate system in which to test functionally the links between inter-species changes in gene regulation and differences in phenotypes. Admittedly, even under the best-case scenario, it may only be possible to focus on cellular phenotypes. Yet, the wide range of cell types that can potentially be derived from iPSCs (for example, hepatocytes, cardiomyocytes and neurons) will offer a range of molecular phenotypes to choose from, perhaps finally making a reality detailed mechanistic functional studies of gene expression evolution in primates.

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