Harnessing mobile genetic elements to explore gene regulation

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Sequences that regulate expression of a gene in *cis* but are located at large distances along the DNA from the gene, as found with most developmentally regulated genes in higher vertebrates, are difficult to identify if those sequences are not conserved across species. Mutating suspected gene-regulatory sequences to alter expression then becomes a hit-or-miss affair. The relaxed specificity of transposon insertions offers an opportunity to develop alternate strategies, to scan in an unbiased manner, pieces of chromosomal DNA cloned in BACs for transcription enhancing elements. This article illustrates how insertions of Tn10 with enhancer-traps into BAC DNA containing the gene, and its germ-line expression in zebrafish, have identified distal regulatory elements functionally. Transposition of Tn10 first introduces the enhancer-trap with a loxP site randomly into BAC DNA. Cre-recombination between the inserted loxP and the loxP endogenous to a BAC-end positions the enhancer-trap to the newly created truncated end of BAC DNA. The procedure generates a library of integration-ready enhancer-trap BACs with progressive truncations from an end in a single experiment. Individual enhancer-trap BACs from the library can be evaluated functionally in zebrafish or mice. Furthermore, the ability to readily alter sequences in a small transposon plasmid containing a regulatory domain of the gene allows re-introduction of altered parts of a BAC back into itself. It serves as a useful strategy to functionally dissect multiple discontinuous regulatory domains of a gene quickly. These methodologies have been successfully used in identifying novel regulatory domains of the Amyloid Precursor Protein (*appb*) gene in zebrafish, and provided important clues for regulation of the gene in humans.

Background

Traditional applications of mobile DNA

Before the advent of modern recombinant DNA technology, mobile genetic elements served a pivotal role as biological

mutagens to identify genes through mutagenesis. Since then they have been used increasingly as a delivery vehicle to introduce exogenous DNA sequences into the germline of an organism with diverse objectives. The introduced sequences often encode proteins whose expression helps monitor the activity of chromosome regions around them. Thus expression of a promoter-less lactose gene was used to assess the activity of numerous *E. coli* promoters through insertion of a Mu phage randomly into the bacterial chromosome.[1] Genes activated in response to a variety of stimuli, such as exposure to DNA damaging agents, were identified using bacterial libraries generated by random insertions of a reporter gene-containing mobile element throughout their genome.[2] In higher eucaryotes, transcription-enhancing DNA sequences located *distal* to gene promoters have been identified by insertion of what are commonly called enhancer-traps. In its simplest form, the enhancer-trap comprises of a basal-promoter driven reporter gene mounted on a mobile genetic element specific to the organism (illustrated schematically in **Fig. 1**). The recombinase/ transposase gene that mediates insertion of the trap into the germ-line is removed from within its functional ends to render it immobile after transposition. In the absence of an expression-enhancing environment the reporter gene within the mobile element is minimally expressed. However the reporter swings into expression mode once it comes under the influence of an enhancer. Thus in situ detection of genomic elements that regulate transcription in *Drosophila melanogaster* used expression of the *E. coli* lacZ gene incorporated into the mobile P-element. Insertions of P-element into the germ-line of flies helped screen sequences that could act at a distance along the chromosome to stimulate expression.[3] Some enhancers are active only in a specific tissue, and can be used to identify specific cell-types: the P-lacZ fusion gene has also helped generate a wide variety of cell-type-specific markers in *Drosophila melanogaster*. Many developmentally regulated genes in *Drosophila* and mice have been isolated and characterized using such enhancer-traps.[4,5] Other species-specific transposon systems modulated to perform enhancer-trapping include the "Sleeping Beauty" transposon-based transgenesis cassette. It established an enhancer-trapping technique for use in vertebrate model systems such as the medaka and the zebrafish *Danio rerio*.[6,7]

Retroviruses represent a group of mobile genetic elements that have proved very useful for trapping enhancers. They require disguising through pseudo-typing to get around their host range
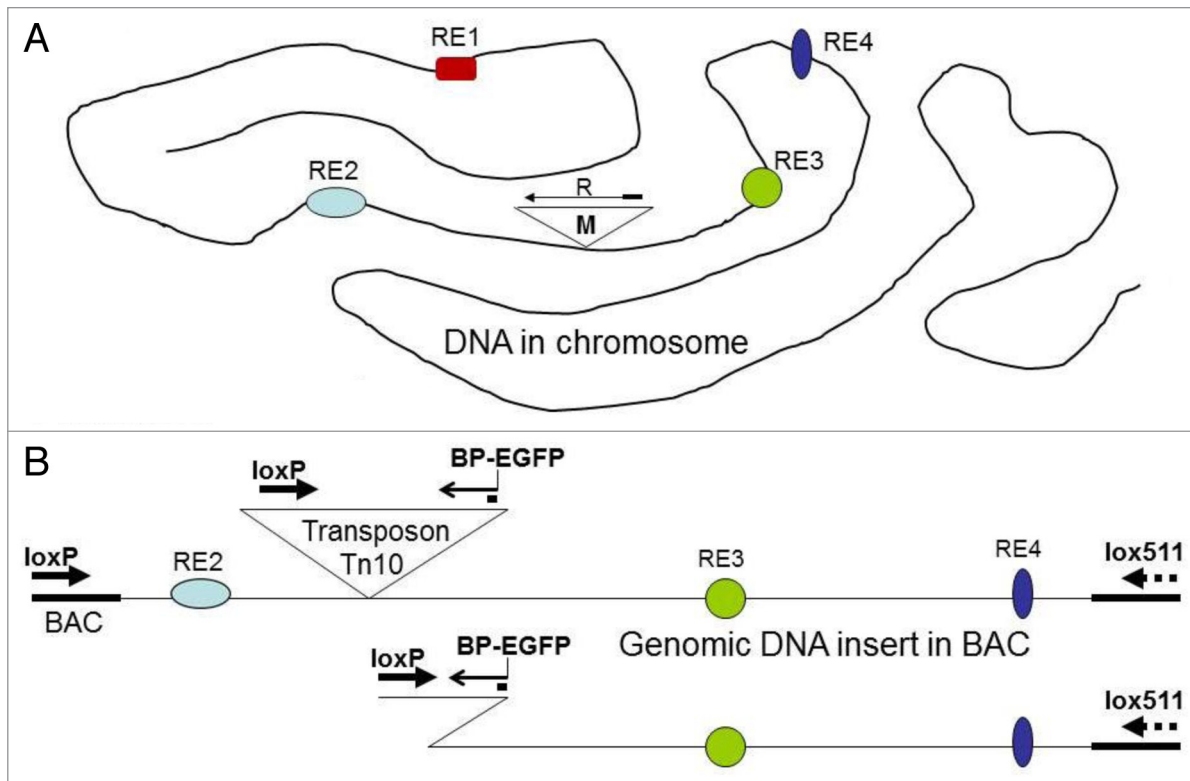
Figure 1. Schematic representation of enhancer-trapping. (**A**) Traditional enhancer-trapping. DNA in the chromosome of an organism is represented by the crooked line. RE1, RE2, RE3 and RE4 are sequences on the DNA that represent Regulatory Elements. The inverted triangle "M" is a mobile genetic element from the organism that has been engineered to render it immobile after insertion, and carries a basal-promoter driven reporter gene "R" within its functional ends. It constitutes the enhancer-trap. Insertion of the enhancer-trap into chromosomal DNA places it in *cis* with other regulatory sequences (RE's) of a cellular gene, and allows the reporter-gene in enhancer-trap to be expressed under the influence of the RE's of the cellular gene. (**B**) Enhancer-trapping using BACs. A fragment of the same chromosomal DNA with RE2, RE3 and RE4 is shown in a BAC. The inverted triangle represents a Tn10 mini-transposon that contains within its 70 bp inverted repeats, a loxP sequence and a basal-promoter driven EGFP gene, shown as BP-EGFP. This constitutes the enhancer-trap. The chromosomal DNA in the BAC is flanked by a loxP and a lox511 sequence. Insertion of the Tn10 into BAC DNA allows the loxP site in Tn10 to recombine with the loxP site endogenous to BAC, and if both are in the same orientation, delete the intervening DNA. The insertion-cum end-deletion process occurs in *E. coli*, and brings the enhancer-trap in *cis* with regulatory sequences (RE3, RE4) of the cellular gene. Upon integration of the enhancer-trap BAC DNA into the germ-line of the organism, the EGFP gene in enhancer-trap is expressed under the influence of the RE's of the cellular gene.

specificities to be useful in heterologous species. Thus a pseudo-typed retroviral vector was used in zebrafish to obtain integration and germ-line transmission of the reporter gene in the enhancer-trap.[8] The approach has also been used with a variety of heterologous cell lines.[9] Injection of retrovirus into de-chorionated fertilized embryos enabled investigators to isolate large numbers of zebrafish lines with germ-line integrated enhancer-traps.[10] Traditional enhancer-trapping of this type has no doubt offered high-throughput screening formats to isolate numerous enhancer-trap reporter lines in the animal systems used for such studies. However the method requires the trap to be introduced into the fertilized egg at the one-cell stage to obtain germ-line expression. Accessibility of the genome for insertion of the trap is limited and not uniform at this stage of development of the embryo and constitutes a potential hurdle. Some regions are more accessible than others, and because one cannot have multiple insertions of the same trap for an unambiguous analysis, much of the genome remains unreachable by this approach. Consequently enhancers have been identified in only a small fraction of the genome in

animals studied, and vast regions appear refractory to enhancer-trapping by such traditional means. Additionally, approaches such as this do not lend themselves easily to identifying and/or addressing questions on mechanisms of how multiple enhancers in domains that may be discontinuous along the DNA act in concert to restrict expression of the gene in a particular tissue. This review highlights an alternate way to use mobile genetic elements to address the complex regulation of expression of genes in higher vertebrates.

Potential hurdles to exploring regulation of gene expression in higher vertebrates

Although gene regulatory elements in vertebrates also encompass non-coding RNA, splicing elements and sequences that mark chromatin structure, here we limit our discussion only to those that bind regulatory proteins to direct expression of a gene often in a tissue- and time-specific manner. A concerted worldwide effort during the previous decade led to the availability of whole genome sequences of a wide variety of organisms, including the human. Sequence comparisons across vertebrate species have

led to the identification of highly conserved sequence patches in non-coding DNA in genomes.[11] Testing the expression of reporter genes directed by these highly conserved non-coding elements (CNEs) was crucial for determining their tissue of function. Transient expression studies, conducted mostly in zebrafish and some in mice, elucidated the importance of such CNE-enhancer elements in regulating expression of genes mostly under developmental control.[12-14] Several new vector systems, containing the inverted repeat sequences of the vertebrate transposon Tol2, were designed specifically for getting reporter gene-CNE fusions expressed from integrated copies in the germ-line of zebrafish.[15-17] Expression analyses indicated that many of these transcription-enhancing CNE-sequences were located tens of thousands of base pairs removed along the DNA from the start sites of transcription of the gene.[12] It is important to note however, that genes expressed from small plasmids integrated into the germline also frequently suffer from position effects. Thus depending on the chromosomal environment, both gene silencing and altered expression can occur after transgenesis.[18,19] To circumvent this hurdle, both "knock-in" technology using homologous recombination,[18] as well as efficient site-specific transgenesis using mobile genetic elements such as the Cre-loxP sites,[20,21] or the PhiC31 integrase system,[22,23] have been developed. Such developments allowed vast numbers of tissue-specific enhancers to be quickly characterized functionally using these procedures. However it is important to note that such studies largely scored individual elements directing/ enhancing expression; and that too outside of their native contexts in the chromosome.

It has become increasingly clear however that the tissue-specificity of an isolated enhancer tested out of its native context may be quite different from that in the context of its own gene.[24] The importance of testing gene regulatory elements in their native context with large stretches of surrounding DNA sequences to capture their complete functional profile was recognized early.[25] Thus Bacterial Artificial Chromosomes, (BACs), originally developed for genome sequencing projects,[26-28] became a viable resource for functional analyses of regulatory elements located distantly from the gene. However, modifications of sequence in the large 300 kb DNA of a BAC could not be conducted using traditional tools of recombinant DNA technology, ie. cutting with restriction enzymes and re-joining with ligase, because the sites are too numerous. Thus a variety of DNA recombination reactions became the tools of choice to alter sequences in BACs. Two of these methodologies developed use homologous recombination, while the third approach is based on the sequential use of a series of mobile genetic elements.

Methodology for Exploring Regulation of Gene Expression in Higher Vertebrates

Using homologous recombination for altering sequences in BACs

In the first approach the major recombination function of *E.coli*, RecA, was re-introduced into the host bacterium DH10B which was made severely deficient in recombination in order to propagate vertebrate DNA.[29] Large amounts of repetitive sequences, considered to be fossils of inactive mobile genetic elements, exist in vertebrate DNA and low recombination activity in DH10B was necessary to prevent the BAC DNA from rearranging. The second approach introduced the recombination functions of phage λ, namely red α, red β and red γ into DH10B. It used shorter homologous sequences for recombination to introduce exogenous DNA cassettes into BACs.[30] Both methods have been widely used to engineer sequences in BAC DNA. They include introducing reporter gene cassettes in frame into the first exon of the target gene, mutating sequences at a chosen site, and introducing loxP sequences.[31-41] BACs containing the β-globin gene were functionalized with EGFP reporter gene using RedET-recombination and expressed in stable erythropoietic cell lines.[36] RecA-mediated homologous recombination was used to introduce a LacZ reporter gene cassette into the Gdf6 transcription unit and long-range regulatory sequences mapped using a series of BAC deletions in transgenic mice.[37] A set of 97 mixed CAA-CAG repeats was introduced into the human htt gene in a BAC by RecA mediated recombination to build a mouse model of Huntingtin's disease.[40] Human BAC clones with 1 kb deletions made by RedET-recombination were used in transgenic mice to functionally identify cell lineage-specific regulatory elements 30 kb upstream of the IFNG gene.[41] BAC modification procedures of moderate throughput and using RedET recombination to introduce the GFP cassette and iTol2 for integration into the zebrafish germ-line have been described.[42] Methodology to construct transgenic animals with functionalized BACs in mice,[43] or zebrafish,[44] have been reviewed recently. Because modifications are targeted in the BAC, initiated one alteration at a time, both methodologies are most useful when clues for specific sequence changes leading to altered function are available. However such hints do not always exist, for example when there is no conservation of sequence across species in regulatory domains of a gene with similar function.[45-48]

Functionalizing BACs with mobile genetic elements: Tn10 transposition followed by progressive end-deletions place exogenous DNA at BAC-ends

A different way to introduce sequences in BACs also uses recombination but does not rely upon sequence homology between vector DNA and genomic inserts of BACs. Mobile genetic elements such as the transposon Tn10 can introduce exogenous DNA, including lox sites, at random locations in BACs.[49,50] Procedures for deleting DNA sequentially from the ends of cloned genomic inserts had been developed earlier using several other transposon systems, but are limited in scope because those methods necessitated additional steps of modifications and sub-cloning of the BAC DNA.[51,52] The loxP-Tn10 approach does not require sub-cloning genomic DNA and it can be used directly on all BACs in the public domain.

The recombination machinery from a different mobile element, namely the Cre-lox system of phage P1, is then used to position the exogenous DNA randomly inserted by Tn10 precisely to the ends of genomic DNA inserts in BACs.[53-55] Deletion of BAC DNA from the site of insertion of loxP-Tn10 to the loxP end of genomic insert DNA makes this possible (**Fig. 1**). The
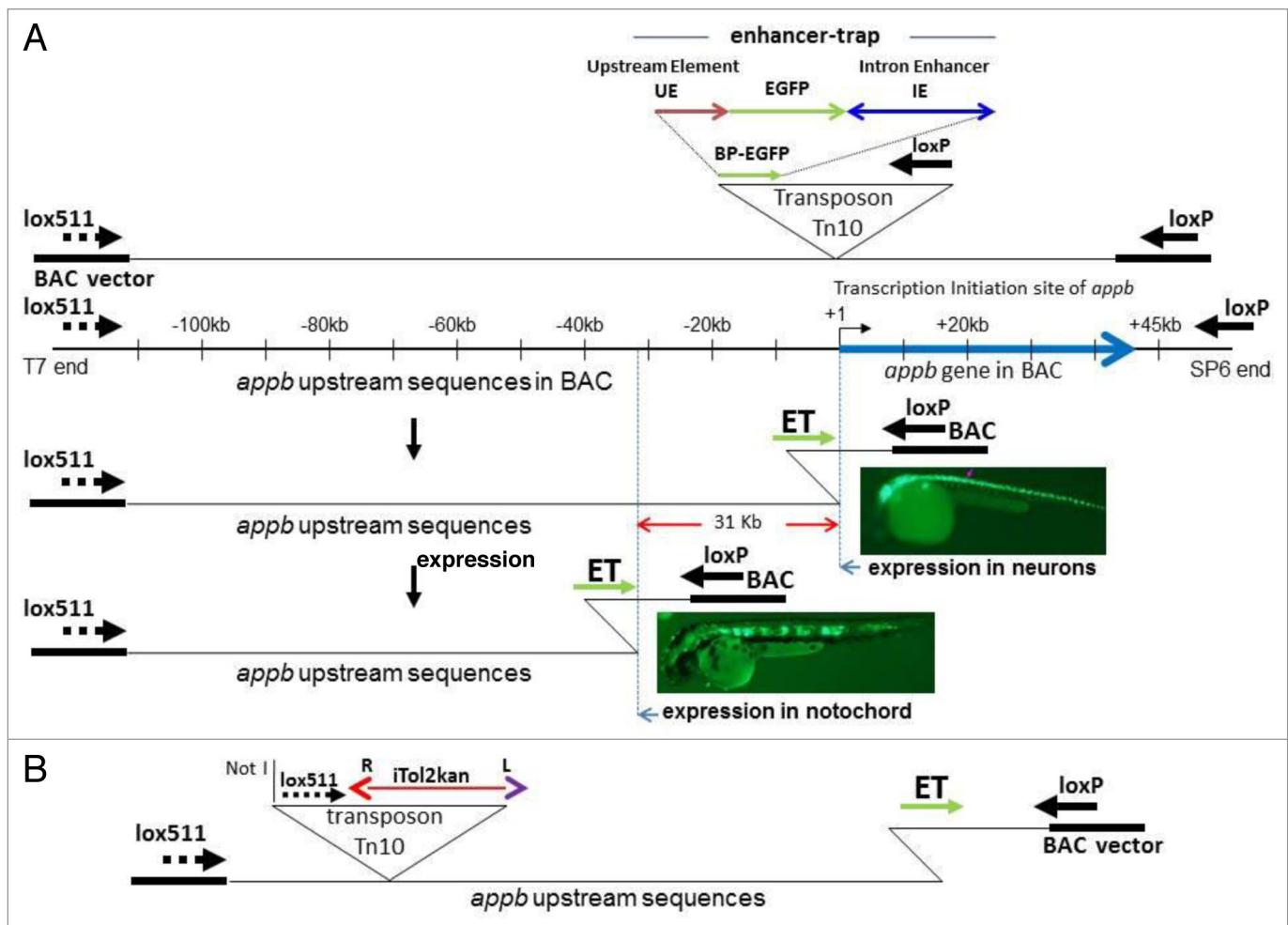
Figure 2. Schematic representation of enhancer-trapping using BACs containing the zebrafish *appb* gene. (**A**) An enhancer-trap comprising of a basal promoter EGFP gene flanked by 0.3 kb DNA immediately upstream of *appb* (UE), and 0.8 kb DNA containing the intron 1 enhancer (IE), was placed in front of the loxP sequence in the Tn10 transposon. Insertions of this enhancer-trap transposon into *appb* BACs and Cre-recombination generate libraries of BACs with DNA progressively deleted from the loxP end with the EGFP enhancer-trap, (ET), at the newly created end. After characterization, suitable BACs from the library are expressed individually in zebrafish embryos. Expression analysis of a large number of enhancer-trap *appb* BACs indicates that ~31 kb of DNA immediately upstream of the *appb* transcription start site is important for neuronal expression of *appb*: in its presence expression is in neurons (inset), while in its absence expression switches to the notochord of zebrafish (inset). Injection of enhancer-trap transposon plasmid DNA itself, without the BAC, also gives this notochord expression pattern.[54,58] (**B**) Schematic representation of insertion of the Tnlox511-iTol2kan transposon into enhancer-trap BAC DNA: The iTol2 cassette is located in front of the lox511 arrowhead. Insertion of ends of the vertebrate transposon iTol2 renders these enhancer-trap BACs integration ready.[55] The red and purple arrowheads pointing outward in the iTol2kan cassette correspond to the 200 bp inverted repeat end R and 150 bp inverted repeat end L of the Tol2 transposon.

loxP sequence, placed within the 70 base inverted repeat ends of the Tn10 mini-transposon, not only helps truncate the intervening DNA between the insertion site and the loxP-end of BACs but also places the exogenous DNA cassette at the newly created end (see refs. 56 and 57 for details). Not all sequences transposed into BAC DNA by Tn10 survive the Cre-lox recombination, as illustrated in **Figures 1 and 2**. The Cre-lox recombination reaction has directionality, and only sequence in front of the loxP or lox511 arrowheads in the Tn10 transposon are retained at the newly created end of BAC DNA, while sequence behind the arrowheads are lost.[56,57] This key feature of the BAC end-deletion procedure is utilized in the diverse applications noted below. Half of all insertions can create deletions because the loxP in Tn10 and the one endogenous to the BAC need to be in the same

orientation. Because P1 phage heads have packaging capacity of ~110 kb of DNA, employing P1-headful packaging in the procedure helps isolate only the deletions if the starting BAC is greater than ~110 kb. Deletions from the opposite end of BAC inserts can be made identically using a lox511-Tn10.[59] Contrary to several earlier reports, cross-recombination between loxP and lox511 does not occur in our approach where Cre protein is synthesized in the bacterial host by a phage P1 infection.[56,59]

Using these procedures libraries of BACs deleted from either the loxP end, the lox511 end or both ends of insert DNA can be generated (refs. 56,57 for details of procedures). Thus in contrast to methods using sequence homology based recombination to engineer end-deletions, which can produce only one alteration at a time, the loxP or lox511 transposon based approach

can generate large collections of end-deleted BACs with exogenous sequence cassettes at the newly created end in a single experiment.

The variety of DNA cassettes introduced into BACs include sequencing primer binding sites, mammalian cell-selectable antibiotic resistance genes, enhancer-traps and sequences specifically recognized by the transposase of another vertebrate transposon system, Tol2.[55] Significantly, the recombinases mediating these transformations, Tn10-transposase and Cre protein, do not act upon sequence repeats and/or other recombinogenic sites in the genomic DNA insert to rearrange it. This particular characteristic makes the approach applicable to a wider variety of BACs, including those containing high levels of repetitive sequences in them (see ref. 53 for an example).

Insertions of Tn10 into BAC DNA from a wide variety of vertebrate genomes appear to be random, demonstrating little sequence specificity for transposition.[53] The reason for this lack of sequence specificity for insertions of a prokaryotic transposon into vertebrate DNA in BACs is unclear, because insertions of Tn10 into prokaryotic DNA have long been known to prefer a somewhat degenerate consensus site.[60] The minor sequence preferences for insertions of Tn10 observed in BACs probably have more to do with the accessibility of sites for Tn10 insertions than specificity for sequences. Note that *E. coli* has histone-like proteins, known as HU protein, which package the vertebrate DNA in the BAC and could modulate accessibility for transposon insertions. Transposition of Tn10 into a rare BAC clone occasionally displays apparent sequence selectivity.[61] However, this was shown to arise from a clonal expansion process from amplifying a single colony of Tn10 plasmid-transformed BAC that had induced the transposase gene prior to actual induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG). Inducing a large pool of Tn10 plasmid-transformed BAC colonies, instead of a single clone, rectifies this potential problem.[61]

Results and Discussion

Using mobile genetic elements to study gene regulation

Truncating BAC ends sequentially with simultaneous delivery of exogenous DNA to the newly created end is integral to the lox-Tn10 approach outlined above and holds promise for a variety of mapping projects. These include locating genetic markers and regulatory elements on physical maps of chromosomes and functionally identifying *distal cis*-acting regulatory sequences located tens of thousands of base-pairs along the DNA from genes. Many of the hurdles to analyzing long-range enhancers by traditional enhancer-trapping, can be overcome by expressing BACs retrofitted with enhancer-traps. Mechanistic questions exploring the role of individual gene-regulatory sequences can be addressed easily using enhancer-trap BACs, while genes with segmented domains of regulation can be dissected functionally. Ability to generate libraries of enhancer-trap BACs in a single experiment makes the approach viable. Expressing a panel of end-deleted BACs from these libraries, individually, in zebrafish has the analogous effect of scanning the entire BAC DNA functionally with

enhancer-traps. The process can be repeated with several overlapping BACs from a contig spanning a chromosome locus.

There is one additional challenge in gene regulation the loxP-Tn10 approach can address. Distribution of CNEs in vertebrate genomes indicate that a substantial number (~30%) of long-range regulators of transcription remain outside the size range of 300 kb BACs to be housed together with the gene it regulates.[12] Thus juxtaposing "far away" gene-regulatory elements located beyond that can be accommodated in BACs by using lox-Tn10 transposons as a delivery vehicle remains a viable alternative. Conceivably, the long range regulatory sequence can be loaded onto a loxP- or lox511-Tn10 transposon and delivered to one or the other end of BAC DNA containing the gene with its other regulators, and tested quickly using our approach. The full potential of this strategy awaits assessment.

Finally, for genes that are essential for survival of the organism, sequence disruptions caused by inserting the trap into the chromosomal copy of the gene or its surrounding DNA during traditional enhancer-trapping may not be permissible. With enhancer-trap BACs, the endogenous copy of the gene in the germ-line is left intact and allowed to function normally.

Mechanism of Regulation of Amyloid Precursor Protein (*appb*) Gene in Zebrafish and Humans

The loxP-Tn10 enhancer-trap technology has been used with BACs containing the *appb* gene as illustrated schematically in **Figure 2**. Well characterized end-deleted BACs from the enhancer-trap libraries were expressed in zebrafish to functionally identify *distal* non-contiguous regulatory elements of the zebrafish *appb* gene. Although further removed from humans than mice evolutionarily, zebrafish has become a popular model system in recent years due to a variety of reasons which include optical transparency of its embryo. The model has been used exhaustively to explore the expression enhancing properties of highly conserved non-coding DNA in vertebrates.[12,13] However, functional non-coding DNA is sometimes not conserved in many developmentally regulated genes including *appb*.[45-48,54] Identifying regulatory DNA domains in the *appb* gene has therefore been a challenge. The enhancer-trap approach is particularly useful in such circumstances because it allows one to scan a series of overlapping BACs containing the gene to identify regulatory sequences without having to guess potential regulatory domains to test. The general location of all regulatory domains, upstream and/or downstream of the transcription start site (TSS), need to be determined at the outset. This was done using the enhancer-trap Tn-US to scan BACs containing the *appb* gene.[54] Tn-US comprised of only the 300 bp DNA immediately upstream of *appb* TSS, fused to the promoter-less EGFP gene in a loxP-Tn10 transposon. Tn-US enhancer-trap BACs gave no expression of EGFP in any tissue. This established the requirement of an *appb* enhancer downstream of the TSS. In the absence of the downstream enhancer in intron 1, there is no expression of a BAC transgene that contained approximately 100 kb of 5′ sequences, indicating that sequences upstream of the TSS alone
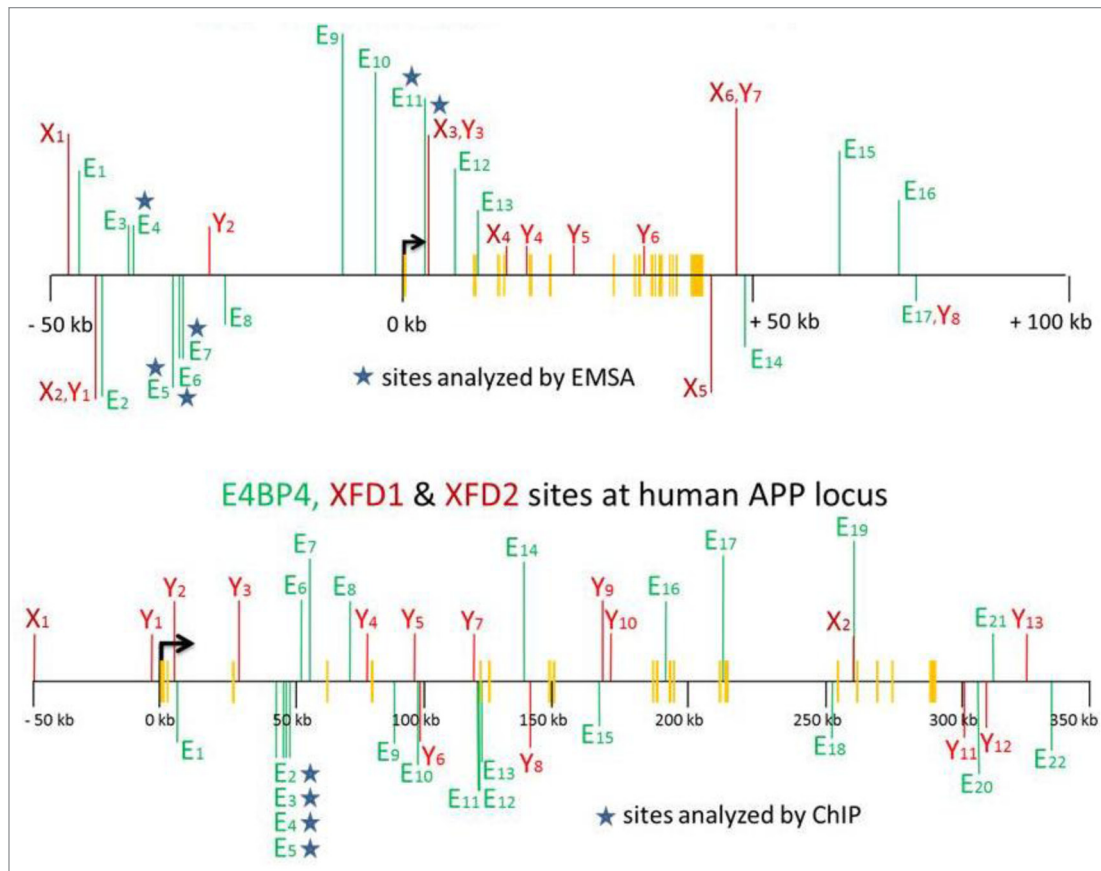
Figure 3. Location of E4BP4 (E, green), XFD1 (X, red) and end-mutated XFD1 sites shown here as XFD2 (Y red), in non-coding DNA in and around the zebrafish *appb* gene (top), and human APP gene (bottom). Green and red vertical lines above or below the horizontal line indicate sites in the forward and reverse strand of DNA, respectively. Short yellow vertical bars indicate exons of *appb* gene in zebrafish of APP gene in humans. The first three exons of human APP are very close to one another near the transcription start site. Stars indicate E4BP4 sites that either bound E4BP4 protein in vitro (zebrafish *appb*), or were marked by H3K9Ac in chromatin immune-precipitation (ChIP) assays with the human cell-line SHSY5Y expressing the APP gene.[58] The bent arrow indicates transcription start site in the two genes.

were insufficient to express the gene. Subsequent enhancer-traps were therefore built to include the minimal intron 1 enhancer element.[54,58]

Transcription Factor E4BP4/ NFIL3 Binding Sites Required for Regulating *appb*

Full-length enhancer-trap BACs built with the minimal intron 1 enhancer expressed *appb* in a pattern similar to the endogenous gene in neurons of zebrafish.[62] However enhancer-trap BACs with intron 1 enhancer deleted for sequences extending until –31 kb, expressed GFP fluorescence specifically in the notochord of zebrafish (**Fig. 2A**). These experiments highlighted the importance of a second regulatory region located between 28 and 31 kb upstream of the gene for guiding *appb* expression specifically to neurons. Deleting this upstream region shifted the expression pattern from being neuron-specific to notochord-specific, which is the default pattern observed with the basal promoter plus intron enhancer combination.[54] Consistent with this finding expression of GFP in neurons of enhancer-trap BACs with a full

complement of upstream sequences reverted to specific expression in the notochord when the three E4BP4 sites at –31 kb of zebrafish *appb* was deleted with a Tnlox511-iTol2kan transposon from the opposite end of BAC DNA (**Fig. 2B**). These upstream sites also bound the E4BP4 DNA binding protein domain, expressed in *E. coli*, efficiently and selectively in vitro.[58]

Functional Dissection of Discontinuous Regulatory Domains is Convenient using Mobile Elements

Regulation of genes expressed during development can be complex, and dissecting the multiple discontinuous DNA domains functionally can be a daunting task.[63] It became clear early on that expression of the zebrafish *appb* gene during development of the embryo involved two discontinuous DNA domains.[54] Functional dissection of such regulatory domains is difficult using other BAC recombineering strategies because potential regulatory sequences to test remained elusive to predict or identify. In the absence of cross-species sequence conservation, as with the *appb* gene, the effort can be extremely

tedious because alterations need to be done one at a time. Mobile elements such as Tn10 greatly facilitate such analyses by helping deliver sequence alterations of certain regions of the BAC, such as sequences in the intron 1 enhancer, back into itself quite efficiently.[58]

Mutating the intron 1 enhancer in enhancer-trap BACs was relatively easy because sequence changes in a small plasmid can be done quickly. The small Tn10 enhancer-trap plasmid containing the intron 1 enhancer was mutated by standard procedures. Each mutated Tn10 was then inserted into *appb* BACs to generate a library of enhancer-trap BACs with a specific mutation in the intron 1 enhancer. A select group of enhancer-trap BACs from 18 such libraries, constructed with numerous mutant intron 1 enhancers was expressed in zebrafish. Expression analyses indicated binding sites of at least two known transcription factors are important for function. They are the clock-regulated immune system modulator transcription factor E4BP4/ NFIL3 and members of the Forkhead gene family (XFD1). A search of non-coding DNA in introns and the 50 kb sequence flanking the *appb* gene for additional binding sites revealed a ~8-fold and ~11-fold greater frequency than statistical of E4BP4 and XFD1 sites, respectively (**Fig. 3**). Although comparison of zebrafish and human APP did not reveal substantially conserved non-coding sequences that could represent regulatory elements, conservation of gene expression via use of the same transcription factors was hypothesized. Support for the proposal comes from the finding that E4BP4/NFIL3 binding sites are over represented also at the human APP locus, as seen in **Figure 3**. Strikingly, one such cluster of four E4BP4 sites in the fourth intron of the human APP gene was marked by a peak of acetylated histones in a human neuroblastoma cell line that expressed APP.[58] Thus it appears that E4BP4/ NFIL3 may regulate human APP expression via binding to distal regulatory sequences. Higher than statistical frequency of E4BP4/ NFIL3 sites have also been noted in both mouse (13 sites), and rat (10 sites) APP (unpublished observations).

Levels of soluble Amyloid Precursor Protein and its peptide fragments have been analyzed as a way to identify patients with incipient Alzheimer disease.[64] A circadian pattern in the variation of β-amyloid levels in mice brains was also observed.[65] It is therefore intriguing to note in this context that transcription factor E4BP4/ NFIL3 has long been known to be clock regulated.[66,67] NFIL3 is also intricately linked with the immune system, where it is required for protecting natural killer (NK) T cells[68] and regulate IL-12 p40 in macrophages.[69] If E4BP4/ NFIL3 were to also regulate human APP expression then it would help us connect in molecular terms the importance of immunological and inflammatory processes that underlie the onset of Alzheimer disease in humans. Thus far, only the pathology and physiology of Alzheimer patients have led clinicians to these conclusions.[70]

Regulation of *appb* in Zebrafish and APP in Humans by the Forkhead Family of Transcription Factors

Some members of the forkhead family of transcription factors, namely fkd1, fkd2 and fkd4, are expressed only in the notochord of zebrafish embryos.[71] This could explain why the expression of EGFP from enhancer-trap *appb*-BACs is exclusive to the notochord for BACs with the three E4BP4/ NFIL3 sites at –31 kb deleted.[54,58] A suppressor role for E4BP4/ NFIL3 is consistent with this scenario and supported by recent reports.[66,69,72] The requirement of the forkhead binding site in intron 1 enhancer for function, and the much higher than statistical frequency of forkhead sites in and around the *appb* gene in zebrafish (represented as X in **Fig. 3**), also suggest a regulatory role for this family of transcription factors.

However fkd sites are not over-represented in human APP. Although there are only two sites with the XFD1 consensus sequence (shown as X in **Fig. 3**), sites with 8 of 9 bases identical (consecutively) to the consensus exist far more abundantly in human APP (13 additional end-mutated sites were identified, designated as XFD2 and represented as Y in **Fig. 3**). It is tempting to speculate that Fkd protein complexes potentially capable of binding to such sites might have evolved in higher vertebrates to accommodate the single end-nucleotide change. Note that XFD2 sites, Y, are less common in zebrafish *appb* (**Fig. 3**). The recently described Crisper-Cas-9 technology should help address whether the end-mutated fkd sites, Y, in the human APP gene are actually required for regulating APP expression in the human neuroblastoma lines.[73]

A novel interplay between Fkd and E4BP4/ NFIL3 is proposed to regulate expression of *appb* specifically to neurons of zebrafish, most likely through other proteins. The findings, if true, would suggest that *appb* in zebrafish and APP in humans follow a similar regulatory logic using the same set of transcription factors, despite a lack of sequence similarity in their regulatory DNA domains. It suggests potential regulatory pathways for the human APP gene may be discovered on the model of conservation of transcription factors.

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