Review

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Enhancers, enhancers – from their discovery to today's universe of transcription enhancers

Abstract: Transcriptional enhancers are short (200–1500 base pairs) DNA segments that are able to dramatically boost transcription from the promoter of a target gene. Originally discovered in simian virus 40 (SV40), a small DNA virus, transcription enhancers were soon also found in immunoglobulin genes and other cellular genes as key determinants of cell-type-specific gene expression. Enhancers can exert their effect over long distances of thousands, even hundreds of thousands of base pairs, either from upstream, downstream, or from within a transcription unit. The number of enhancers in eukaryotic genomes correlates with the complexity of the organism; a typical mammalian gene is likely controlled by several enhancers to fine-tune its expression at different developmental stages, in different cell types and in response to different signaling cues. Here, I provide a personal account of how enhancers were discovered more than 30 years ago, and also address the amazing development of the field since then.

Keywords: cell-type identity; gene regulation; locus control region; super-enhancer; transcription factor; transcriptional enhancer.

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Prologue

Francis Crick once commented that after a new scientific concept is established, it is difficult – if not impossible – to see in the mind, how it was before. This applies perfectly to two phenomena that, in short succession, changed our understanding of eukaryotic gene regulation: introns and enhancers. They were not known in bacteria and no one had ever postulated their existence, simply because there seemed to be no need for them. Now that introns and enhancers are part of the scientific world, one cannot imagine how higher forms of life could ever have evolved without the multitude of tailored proteins that can be produced by alternative splicing, or without the sophisticated patterns of remote transcription control by enhancers. Indeed, the complexity of an organism is primarily determined by the variety of gene regulation mechanisms, rather than by the number of genes.

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The holy grail

In the fall of 1978, I returned to Zurich University from Cold Spring Harbor, following an offer from Max Birnstiel to establish my own group at the Institute of Molecular Biology II. At that time, the discovery of splicing introns had made its way into biology but enhancers were still to come. The elucidation of gene regulation in higher organisms was then considered to be the holy grail of molecular biology, and it was also clear that this grail could only be reached by the use of genetic tests. But how? A few years earlier, the situation had been daunting. In bacterial and bacteriophage genetics, the key step was to cleverly interpret a plethora of random mutations. Compared to prokaryotes, generation times in the mouse, even in the fruit fly *Drosophila*, are discouragingly long, and thus the road to insight appeared endless. But the advent of DNA cloning in bacteria, together with the development of DNA sequencing in the mid 1970s, had changed everything. Suddenly, the genes of higher eukaryotes became open to experimentation. The irony of the new situation was that predetermined mutations, such as a deletion between two restriction cleavage sites, could be made with relative ease in a cloned gene, but it was not at all obvious how to test the outcome. The hope was to introduce a modified gene back into the nucleus, into the chromosomal DNA of a living cell, and assess the consequences. Zurich University was then a hotbed for this new research. Using newly available techniques, Max Birnstiel tested tRNA and

histone genes by injecting them into frog oocytes (Kressmann et al., 1978), and Charles Weissmann stably transformed mouse fibroblasts with a β-globin gene that was linked to a selectable marker, a viral thymidine kinase (Mantei et al., 1979). Each of them created his own term for this new research endeavor: surrogate genetics (Max Birnstiel) and reverse genetics (Charles Weissmann). (For a personal account of early molecular biology in Zurich, see Schaffner, 1999.)

Eukaryotic gene regulation: nothing new?

How was gene activity regulated in higher organisms? Besides the classic bacterial operon with repressor and activator binding in the immediate vicinity of the transcription start, alternative mechanisms also seemed possible. Britten and Davidson (1969), based on the surprising fact that many RNAs never leave the nucleus, proposed that classes of repetitive 'activator RNAs' would induce gene transcription by forming sequence-specific contacts to 'receptor' DNA associated with protein-coding genes. Adding to the mystery were the so-called lampbrush chromosomes observed in oocytes of amphibians and certain insects, whereby all the chromosomes were thickly decorated with their own transcripts. Were the messenger RNAs governing embryonic development processed from these long transcripts? In other words, was there perhaps no regulation of transcription whatsoever? (Curiously, what in our laboratory slang we called at the time the 'transcribe now, think later' model, subsequently turned out to occur in some unicellular eukaryotes, including trypanosomes and *Leishmania*, where gene activity is regulated posttranscriptionally; Clayton, 2002).

When the dense fog that had shrouded the eukaryotes began to lift, the first glimpses seemed to confirm that gene regulation processes were similar to those of bacteria. For example, Robert Tjian, 'Tij', a superb biochemist and lab mate during my Cold Spring Harbor time, found that T antigen, the major early protein of simian virus 40 (SV40), bound to sites at the origin of replication which overlapped its own ('early') promoter region (Tjian, 1978; Rio et al., 1980). This suggested a function analogous to that of a bacterial repressor. Histone genes, unlike most other genes, occur in multiple copies per genome, which facilitated their isolation (Birnstiel et al., 1974). When the Hogness lab sequenced the *Drosophila* histone genes, upstream of their protein-coding regions a common AT-rich motif of consensus TATAAAT panned out; it was later referred to as the Hogness box, or TATA box (Lifton et al., 1978). The sequence of this motif, its defined position some 25 base pairs (bp) upstream of the transcription start (Gluzman et al., 1980; Grosschedl and Birnstiel, 1980a) and its importance for proper transcription (Grosschedl et al., 1981) were strikingly reminiscent of the bacterial TATAAT promoter motif, referred to as Pribnow box or -10 box. It was also known that the eukaryotic 18S and 28S ribosomal RNAs were transcribed from one promoter as a long transcript, like a bacterial operon, and only later processed to individual RNAs (Dawid and Wellauer, 1976; Schibler et al., 1976). In this context it should also be noted that Ed Lewis in his classic analysis of the *Drosophila* bithorax complex, described *cis*-acting mutations in putative repressor binding sites (Lewis, 1978).

Freshly-born PI: what to do?

I decided to study gene regulation in eukaryotes by combining my expertise in histone gene cloning and on simian virus 40 (SV40), stemming from the postdoc times at Zurich University and Cold Spring Harbor, respectively. SV40, a small DNA virus that readily grows in cultured monkey cell lines, was one of the most intensely studied viruses at that time. Like others in the field, I expected that eukaryotic gene regulation would likely mirror the situation in bacteria, though probably more complex with hundreds of repressors and corresponding binding sites in the promoters. I thus decided to start out by dissecting the putative regulatory elements upstream of the coding sequence of a histone gene, by doing what was later referred to as 'promoter bashing', i.e., introducing numerous alterations and observing their effects. In retrospect this appears as a rather conventional project, but back then any kind of information on genes in a higher organism was exciting. There was the feeling of entering new land with the possibility of discovering something unexpected, and to ultimately find out how our own genes tick.

Advantage Berg

I intended to introduce cloned genes for functional tests into mammalian cells, using the calcium phosphate method that had hitherto been used for transfecting viral genomes or for stably transforming cells in culture. Recombinant DNAs were to consist of a vector plasmid, a eukaryotic gene such as a sea urchin histone gene, and an SV40 replicon. Only via viral DNA amplification, I reasoned,

could I hope to get sufficient gene copy numbers for expression analysis; because only a fraction of the cells in a Petri dish would be transfected, i.e., import the foreign DNA into the nucleus. On paper this was a great system: no tedious removal of the vector plasmid, no virus growth required, just DNA amplification and, most importantly, no size constraint for the gene to be tested. All these were advantages over the system of Paul Berg at Stanford who, in an attempt to use SV40 as a vector for gene therapy, had been able to produce β-globin protein in a recombinant virus by driving a globin cDNA minigene with the viral late promoter (Mulligan et al., 1979). However, there was also a disadvantage of some relevance: his system worked, mine did not. In repeated attempts, all of my SV40-based recombinant plasmids stubbornly refused to replicate in monkey host cells, although there was adequate expression of T antigen, the viral replication factor. Was the large size of my constructs the problem? Were sea urchin histone genes incompatible with mammalian cells? Only one clone, a plasmid with three tandem inserts of the SV40 genome, worked – but alas, not as I expected: From the repeats, one complete viral genome popped out by recombination, replicated and produced virus, while the rest stayed behind, silent. This clone, pBSV3x, at least allowed me to turn the defeat into a small victory in another project (see below). Also it dawned on me that the vector plasmid was the culprit that was blocking replication, which of course destroyed the elegance of the system. Later I learned from Mike Botchan, a lab mate from the Cold Spring Harbor times and now at Berkeley, that they had independently found that SV40-type replication was blocked by 'poisonous' sequence elements in plasmid DNA (Lusky and Botchan, 1981; subsequently, I found that the inhibition was less pronounced with other types of plasmids and other cells; whether that insight would have accelerated, or rather delayed, enhancer discovery is difficult to say). Regardless, I found it disquieting that one year had gone by and I still had not gotten further with my main project, even though I was supported by a technician to help with routine work such as buffer and plasmid preparations. Like so many others, I had underestimated the time it takes to get a lab going smoothly. Fortunately, I enjoyed bench work tremendously, in line with the saying that a young group leader might still be his/her own best postdoc for years to come.

Spreading out in three directions

A tendency for tinkering and following up unexpected results was at the same time my strength and weakness. Indeed there was success on a side project: Out of curiosity I once tested whether recombinant plasmid DNA could be transferred directly from bacteria to mammalian cells, without the detour of plasmid isolation and purification. The above-mentioned pBSV3x plasmid came in handy for this, because even a single positive event in a culture dish would ultimately reveal itself due to the spread of a viral infection. Lo and behold it worked! Optimization of the technique followed, and the publication (Schaffner, 1980) gave me something to justify my existence. Because a given recipient cell could take up many identical plasmids from a single bacterium, this method was later adopted by a biotech company for the isolation of specific mammalian genes via expression selection.

When I realized that Max Birnstiel also intended to study the transcriptional regulation of his favorite histone genes, I resorted to another gene that I had unduly left waiting in the fridge for some time. During a visit to Caltech in spring 1978, I had met Tom Maniatis and asked, somewhat hesitantly, for a clone of the complete rabbit β-globin gene that, unlike a cDNA, would include all the regulatory promoter sequences. Red hot stuff – it was the first mammalian gene isolated! To my delight, Tom agreed instantly, generously provided DNA clones and all relevant information without a request for co-authorship or other strings attached – and this even a few months before publication (Maniatis et al., 1978).

This change of test system came timely for ambitious Sandro Rusconi, my very first PhD student, who was eager to generate transgenic frogs and mice (for portraits of group members, see Figure 1). We reasoned that a hemoglobin component would be easier to detect and more likely to be tolerated by the host organism than a sea urchin protein – also the prospect of producing a mammalian protein was more attractive. For Sandro, obstacles of any size were a welcome challenge, and he soon got promising results by injecting the gene into fertilized eggs of the clawed frog *Xenopus laevis*. The origin of this latter project seems worth mentioning, an illustration of how in science (and elsewhere) one can miss a concept that in retrospect appears blatantly obvious. At that time I commuted by train and often conversed with passengers in the same compartment, sometimes praising enthusiastically the wonders of molecular research. Once I also mentioned DNA injection into frog oocytes, then the favored technique in the Birnstiel group (a living test tube, according to Max), and the response was: 'And what happens later – are these frogs turning into part-sea urchins?' Wow – yes – frogs that carry a foreign gene! How exciting! Strange as it sounds, up to then neither I nor the Birnstiel people had considered

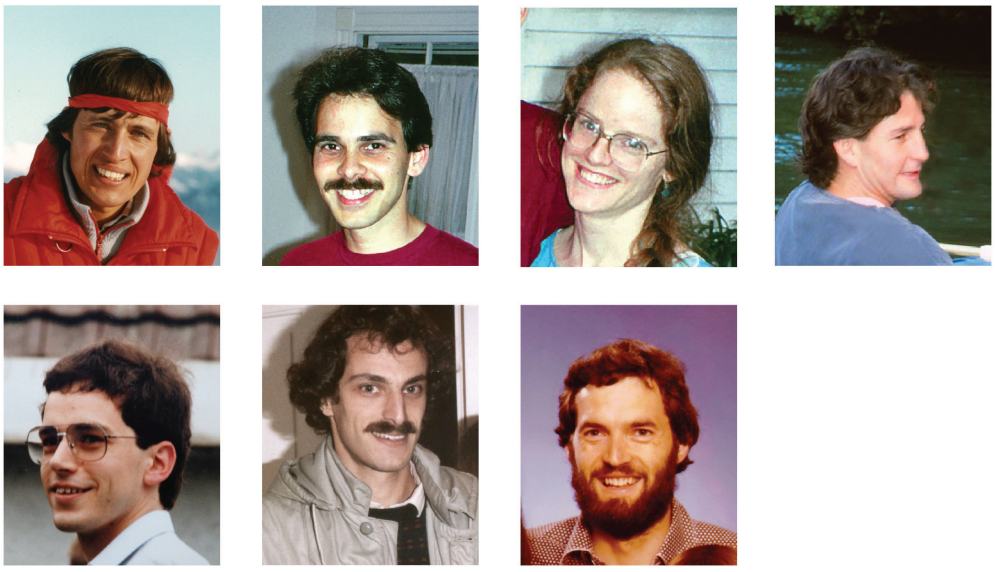

Figure 1: The protagonists in the Zurich enhancer group of the early 1980's. Top row from left to right: Sandro Rusconi, Julian Banerji, Laura Olson, Jean de Villiers. Bottom row from left to right: Frank Weber, Didier Picard, Walter Schaffner. (For their careers, see Acknowledgements.)

following the fate of the injected genes through frog development, i.e., to produce transgenic animals.

A big effect, met with suspicion

To progress with my original project, the regulation of gene expression, I selected a line of HeLa cells that was easily transfectable, and also hoped that a newly obtained antibody for β-globin might be sensitive enough to reveal at least some globin expression in recipient cells. A 5000 bp fragment of rabbit DNA harboring the β-globin gene was inserted via a unique KpnI cleavage site into two vector plasmids, one of which also contained an incomplete SV40 genome (Figure 2A,B). Why SV40, as it had not worked for amplification? Simply for convenience: I had a large batch of this plasmid, and the expression of T antigen would give a clue to the transfection efficiency. To my delight, when I tested both constructs in HeLa cells, β-globin was detectable by immunofluorescence – but only with the construct that contained SV40 DNA.

Skeptical, I assumed some kind of error in the inactive recombinant – perhaps a globin mutation had inadvertently been generated upon sub-cloning? To test this, the globin gene was excised from that plasmid and cloned again into the SV40-containing vector (a legacy of my PhD training: don't fool yourself, mind the controls). On January 15 1980, in the dark microscope room, it was clear: globin expression was indeed very strong, but only when the gene was linked to a segment of SV40 DNA! A strange but robust effect on gene expression was evident – and I was the lucky one to find it! My next thought, admittedly rather prosaic, was: 'Great, this will keep me busy for a year!' Because the construct did not replicate, an effect of gene copy number was excluded. The mind-blowing boost of globin expression brought about by the linked SV40 DNA turned out to be highly reproducible, irrespective of the orientation of the globin insert at the KpnI cloning site, and with different constructs. Nevertheless, nagging doubts lingered for several months. Was it really genuine stimulation of globin transcription, as I assumed, or, far more trivial, just read-through from a viral promoter? (As the boosting effect was independent of globin gene orientation, the latter seemed less likely). The possibility that the SV40 regulatory region encoded a short protein that stimulated globin mRNA translation was excluded by the finding that the viral DNA only worked *in cis*, i.e., not from a separate co-transfected molecule.

So far, all results were based on semi-quantitative immunofluorescence. Sandro Rusconi, who, as mentioned, was on his way to producing transgenic animals with the rabbit β-globin gene, diverted some time to my project and in summer 1980 did a crucial transcript mapping with S1 nuclease. He found that the active segment of SV40 DNA enhanced the level of transcripts from the genuine globin initiation site by at least 100-fold! A spinoff technique from all this was the transient transfection assay. Its novelty deserved a publication of its own, which I failed to appreciate at the time.

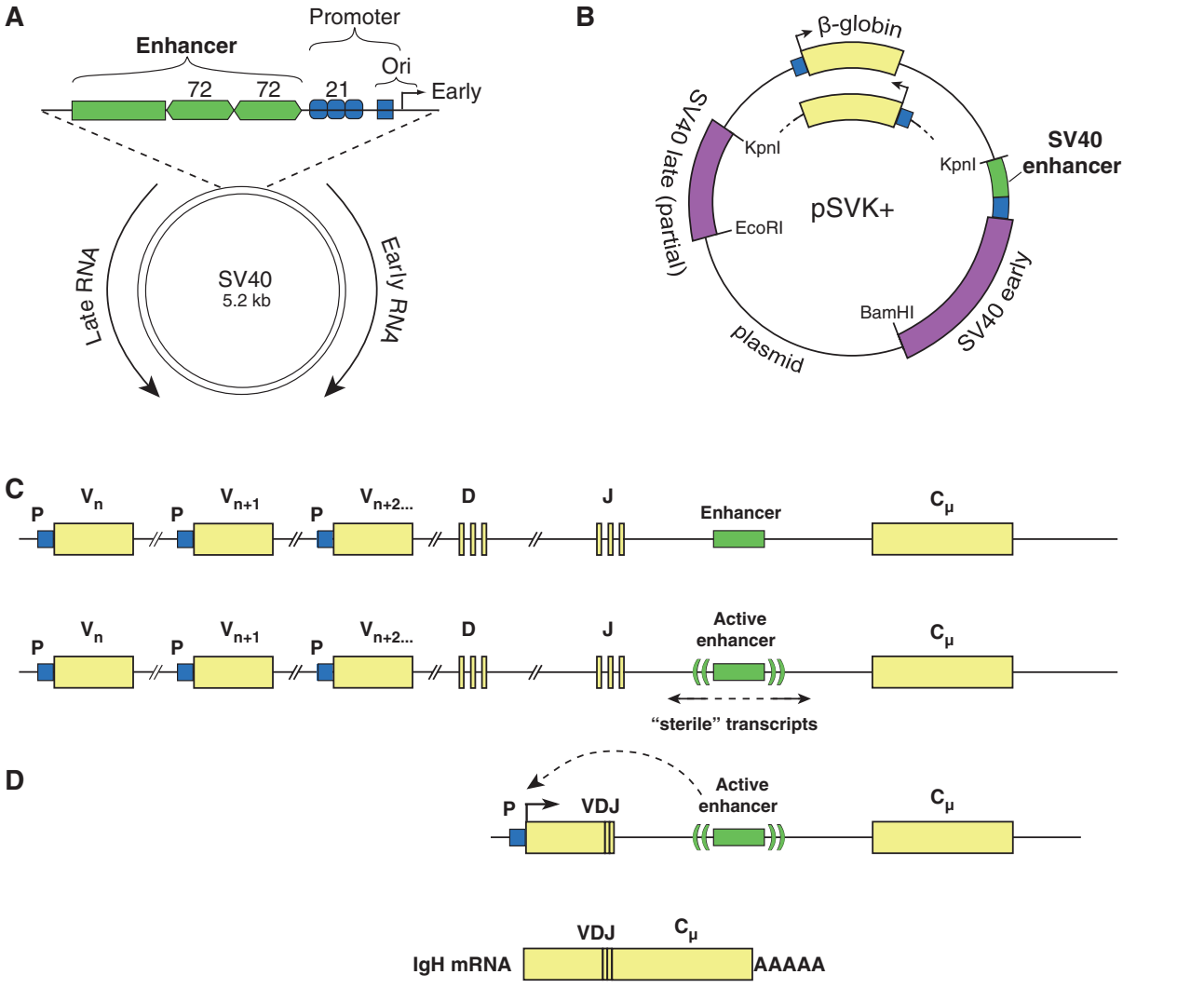

Figure 2: The first viral and cellular enhancers.
(A) Schematic view of the non-coding control region of simian virus 40 (SV40) from which the first transcription enhancer (in green) was characterized (Banerji et al., 1981; Moreau et al., 1981). The early promoter region (in blue) contains three-fold repeated binding sites for the transcription factor Sp1 (Dynan and Tjian, 1983) and an AT-rich segment with a TATA box for binding of other transcription-relevant proteins. Even though enhancer and promoter elements may overlap, the activity of DNA-binding transcription factors can be specific; here, Sp1 preferentially activates transcription from a proximal ('promoter') position (Seipel et al., 1992). The duplication of the 72 base pairs (bp) segment, typical for the enhancer of SV40 laboratory strains, represents an adaptation to fast growth in tissue culture owing to increased early gene expression; the archetypical virus from macaques has only one 72 bp segment (Ilynskii et al., 1992). The duplication also illustrates a correlation between enhancer size and activity (Schaffner et al., 1988). (B) The recombinant plasmid (pBSVK+) that led to the discovery of the enhancer effect. It contained a β-globin transcription unit inserted at the KpnI cleavage site into an incomplete SV40 genome. When this recombinant and a similar one lacking SV40 DNA were transfected side-by-side into human HeLa cells, β-globin expression was detectable by antibody-based immunofluorescence – but only in the construct with SV40 DNA. The same enhanced expression was observed with the twin plasmid pBSVK-, which contained the globin gene insert in opposite orientation, as indicated inside the circle. Subsequent quantification of globin mRNA revealed a 200-fold stimulation of globin transcripts by SV40 DNA (Banerji et al., 1981). (C) The first cellular, rather than viral, transcription enhancer (green) was identified in the immunoglobulin heavy chain (IgH) locus preceding the coding sequence for the constant part Cμ. Upon its activation the IgH enhancer, like many if not all enhancers, induces some local transcription. Such transcripts, dubbed enhancer RNAs (eRNAs) (Kim et al., 2010; Andersson et al., 2014), can also include RNAs with a regulatory function (Orom and Shiekhattar, 2013). (D) After somatic IgH recombination, which links one of the V (variable) regions via a diversity (D) and a joining (J) element to Cμ, the enhancer is located in the intron between VDJ and Cμ and directs transcription of the IgH M chain mRNA (scheme below); (modified from Banerji et al., 1983).

Taken together, these results meant nothing less than that the SV40 'enhancing' DNA segment was able to boost transcription independent of its orientation and at distances of more than 1000 bp from a (related or unrelated) target promoter! And it even worked from a position downstream of the transcription unit. These properties were subsequently generally accepted as an enhancer definition.

At the 2nd International Congress on Cell Biology in Berlin in late summer 1980 I was scheduled for a talk in one of the parallel sessions and decided on short notice to report the enhancer effect. My presentation was met with apparently modest interest, underlined by some polite hand-clapping, a far cry from the bombshell I had dreamed of. Was the finding too alien to be accepted at once? Or was I simply too nervous to give a convincing presentation? Fortunately Phil Sharp, the co-discoverer of splicing, was part of the audience; he approached me afterwards, radiant, and called it 'the discovery of the year'. 'Thank you! but yours is the discovery of the decade', I replied.

A bunch of characters

After showing that SV40-induced transcripts started at the correct globin 5′ end, Sandro returned to transgenics, while the SV40/globin project was continued by Julian Banerji, a new PhD student from Stony Brook. I had met him at Cold Spring Harbor where he impressed me as bright and curious, thus I offered him to come to Zurich. He was followed by his undergraduate girlfriend Laura Olson. As she was not yet eligible for a PhD program we employed her as a lab assistant and did not regret it – she turned out to be a reliable experimenter and great team member.

Julian was the only one who could design a complete cloning scheme of several steps without pen and paper, just in his head. Before others might have finished drawing a plan, he had already made the plasmid construct and variants of it, to be tested in a few blitz experiments. From the masses of data generated in intense bouts of activity, the correct results could be deduced. Julian was a great asset to the lab and basically a goodhearted fellow – but his critical attitude towards official authorities and blunt comments did not go down well with everybody. I myself was not a talented organizer, to say the least, but whatever I was and did, Julian did too, but more extremely. He would show up at noon or later but then, with an irregular mix of reading science, gossip, dedicated bench work and an occasional snack, easily stay till 6 am the following day.

And there were more PhD students knocking on my door: Jean de Villiers from South Africa and the ultra-Swiss Frank Weber from the ETH nearby. Jean, who soon found in Julian a good buddy, was similarly outspoken, with comments on subprime scientific presentations such as 'And the take-home message is: If you can't convince them, confuse them.' He was to be very productive, especially when considering that he also moonlighted as a male fashion model and was dating his newly-found Swedish girlfriend. Frank was an extremely skilled and efficient experimenter with a no-nonsense attitude – but a lost cause when it came to going for beers in a crammed bar. His clockwork 8-to-6 work discipline without any coffee breaks and time for chit-chat was met with a curious mix of playful derision and baffled admiration by Julian and Jean. At times I found it a challenge to integrate all these guys, each a primadonna in his own right, into a functioning team.

A new era

In the meantime Pierre Chambon (Strasbourg) who, like Max Birnstiel and Charles Weissmann, was a towering figure in Europe's early molecular biology scene, was studying SV40 and ovalbumin genes. In autumn 1980 he co-organized a meeting at Seillac Castle, France. The prevailing view at the time was that eukaryotic genes were controlled by promoters with a local influence, limited to about 100 bp from the initiation site, but this era was definitely to come to an end. At the castle, Max Birnstiel reported their studies on tRNA gene transcription and the finding of a 'modulator' DNA segment in the histone H2A gene of a sea urchin, located between -110 and -450 bp from the transcription start. Deletion severely reduced transcriptional activity by more than one order of magnitude; surprisingly inversion of the segment, anticipated as a control, made it work even better! This was to be the first published case of a regulatory DNA sequence of a higher organism with activity in either orientation (Grosschedl and Birnstiel, 1980b). Pierre Chambon and Christophe Benoist reported that SV40 early gene transcription depended on a segment between positions -100 and -260 that contained the so-called 72 bp repeats (Figure 2A)(Benoist and Chambon, 1981; see also Gruss et al., 1981). I presented the SV40 enhancer effect, for the second time after the Cell Biology Congress in Berlin; this time it received more attention. In the coffee break Pierre and I discussed our findings, and we agreed that he would test activation at a distance, which obviously had

not occurred to him in their deletion studies, while we, having narrowed the activity to a segment with the SV40 non-coding control region, were going to see whether his 72 bp repeats contained the essence of enhancer activity. Julian Banerji was instrumental in consolidating and refining the story. He showed that the 72 bp repeats indeed represent a major part of the enhancer, also exploiting some deletion mutants kindly provided by Yasha Gluzman (Cold Spring Harbor), who had produced them for other purposes. In one of Julian's constructs, when the enhancer was flanked on both sides by plasmid DNA, the activity was severely reduced. This effect convinced us of the previously observed 'activation from downstream': in our circular recombinant DNAs (Figure 2B), an enhancer position downstream of the globin gene could also be considered to be far upstream of it; however, transmission in the latter case would have had to work through bacterial plasmid DNA, which was inhibitory.

I mailed a manuscript to Mike Botchan, to whom I owe most of what I will ever know about SV40. His response was enthusiastic: he had read the story 'with bated breath'. 'Why don't we call your enhancer the Schaffner box', he suggested. Although I was immensely flattered for a moment – after all a Hogness box, a Shine-Dalgarno sequence, a Pribnow box were already in circulation – I dismissed the idea (a case of thinking-outside-the-box, I assume) because it would have made light of the contributions of Julian and Sandro. Instead, I used another term. Up to then I had referred to the enhancer effect, or to enhancer-active DNA; in his response Mike simply called it an enhancer, a term that I gladly adopted and that has stuck ever since.

Daring proposals

In parallel to the publication from Pierre Chambon and his colleagues (Moreau et al., 1981) our long overdue paper appeared in Cell (Banerji et al., 1981). I was always eager to extract some biological sense from an observation. But from all the proposals made in our papers, those in our enhancer article were most grand and far-reaching. Yet, amazingly, they have stood the test of time:

An enhancer works in either orientation; it is able to activate a linked gene over long distances, even from a position downstream of the transcription unit. Enhancers are present in unrelated viruses such as retroviruses; viral oncogenesis can be the result of hyperactivation of a cellular regulator gene by the enhancer of a nearby inserted retrovirus genome. In higher organisms, cellular enhancers are activating the genes within each chromosome domain, and classes of different enhancers are involved in the developmental, as well as the tissue-specific, expression of genes (Banerji et al., 1981).

Pierre Chambon and his formidable team – who often left their competitors behind in a cloud of dust – were less lucky in this case. Using two heterologous promoters and protein immunofluorescence as a readout, they also saw cases of an orientation-independent activation over a long distance. However, in a paradigmatic series of constructs with spacer inserts of different length, the effect faded out over about 700 bp, indicating that the enhancer segment was at its best when adjacent to, if not overlapping with, the promoter. A possible explanation for the discrepancy to our long distance effect is that they used plasmid fragments as spacers – DNA which we had found to interfere with the transmission.

While the immunoglobulin enhancer would not have been found without Julian's contribution (see below), the cornerstones of the SV40 enhancer story were already in place when he joined the team. So why was the first enhancer paper Banerji et al., rather than Rusconi et al.? Simple: for a daredevil and technical wizard like Sandro the enhancer effect was not enough. Son of a wine-maker in Ticino, the Italian-speaking part of Switzerland, tall and handsome, a skiing ace, admired by the students in lectures and lab courses; for him the sky was the limit. He wanted to be the first one to cure genetic diseases by gene therapy and to this end started out by genetically modifying frogs and mice. Was he wrong to reach for the stars? It would be unfair to say that he grossly misjudged the importance of the enhancer effect. At that time, mind you, the SV40 enhancer could still have ended up as a peculiar trick of a small DNA virus. Sandro produced a fine PNAS paper on the generation of transgenic *Xenopus* (Rusconi and Schaffner, 1981) but with regard to transgenic mice he was seriously scooped by Frank Ruddle (Gordon et al., 1980). In retrospect (as someone said, hindsight is an exact science) it would have been better for Sandro if he had stayed on to round off the enhancer story. Such are the vagaries of science, and life.

More enhancer properties emerge

How general was the enhancer effect? Jean de Villiers was quick to show that polyoma virus, a distant relative of SV40 in the mouse and founding member of the ever expanding family of polyomaviruses, also contained an enhancer next to the early promoter (de Villiers and

Schaffner, 1981). Later, in a collaboration with Bob Kamen at ICRF London, he found that this enhancer also boosted viral DNA replication (de Villiers et al., 1984). But what made an enhancer tick? Was it a specific DNA segment favorable for docking of RNA polymerase II, from where it would slide to the promoter? To our disappointment, the few established or suspected enhancers known by then could not be aligned to a consensus sequence. Therefore, at one time we considered a peculiar DNA structure for enhancers, rather than a primary sequence, but it became clear quite soon that the efforts to fold them into a unifying self-complementary structure were doomed.

A more complex scenario was suggested by new findings from George Khoury's and our lab, which indicated a species preference of enhancers. In mouse cells, the enhancers of a murine retrovirus and of mouse polyomavirus were highly active, while in monkey cells they were no match for the SV40 enhancer (de Villiers et al., 1982; Laimins et al., 1982). The obvious explanation was that enhancers cooperated with cellular 'factors' such as proteins. Later other labs, notably of Robert Tjian (Berkeley), Albrecht Sippel (Freiburg) and Moshe Yaniv (Paris), provided evidence for proteins binding to promoters and/or enhancers using DNA footprinting, *in vitro* transcription and electrophoretic mobility shift assays (Dynan and Tjian, 1983; Nowock et al., 1985; Piette et al., 1985). Now it is clear that an enhancer is a DNA platform that interacts with a multitude of transcriptional regulatory proteins.

What a strange place to find an enhancer!

The big quest, however, was for a cellular enhancer. During this time Julian Banerji gained further momentum, proposing new experiments every day, if not every hour. Not all were good, of course, and we anyway lacked manpower to pursue all his fancies. But once he made the brilliant suggestion that the best place to look for a cellular enhancer would be the immunoglobulin heavy chain (IgH) gene locus, on which he had done his master's thesis at Stony Brook. The transcription rate at that locus was outrageously high, with RNA polymerases literally transcribing bumper-to-bumper. He constructed a series of plasmids with subsegments of the IgH locus, which I tested in HeLa and monkey CV-1 cells, alas without success. Our last hope was for an enhancer that would reveal itself only in B lymphocyte-type cells, the genuine antibody producer cells. At that time I did a major part of the lab's cell culture work and considered myself an expert, but I repeatedly failed to transfect any B-type myeloma cells using the popular calcium phosphate method. Even the selection of a variant line of these cells that I got to flatten and attach weakly to the culture dish, was to no avail.

Almost one year later I resorted to DEAE dextran, an old and comparably inefficient transfection method, as well as gentler cell fixation. This was the breakthrough; B cells gave a strong signal with the SV40 enhancer and, amazingly, also with one of the segments from the IgH gene! With initial disbelief Julian and I checked again and again the position of the active segment: unlike SV40, where the enhancer was relatively close to the initiation site, if not interwoven with the promoter, the IgH enhancer was located within the intron preceding the constant region, which meant that it activated the promoter from within the transcription unit and only after a productive Ig gene rearrangement (Figure 2C,D). Most importantly, it also meant the immunoglobulin enhancer was cell-type-specific – the first genetic element of its kind! And of course it demonstrated that a downstream enhancer position was a reality and did not only work in synthetic globin-SV40 constructs. What was more, the enhancer could explain why chromosome translocations that brought a c-myc gene into the vicinity of the IgH locus were often observed in leukemia. The latter was an insightful suggestion by Julian – irrespective of the fact that later studies in Michael Neuberger's lab revealed another strong enhancer downstream of the IgH locus as the major culprit in myc deregulation (Pettersson et al., 1990).

Here was one of the very few moments in my life where an experimental result that was at first confusing, in one stroke provided an elegant explanation for several phenomena.

Feeling the breath of competitors

When I presented the cell-type-specific IgH enhancer at a Cold Spring Harbor Workshop on Enhancers in spring 1983, I learned to my dismay that Susumu Tonegawa had obtained essentially the same results. After my return to Zürich I got a phone call from Ben Lewin of *Cell* who invited me to publish our findings in his journal, only to get another call shortly thereafter from Peter Newmark, Editor of *Nature*, with the same offer. Not only had the rumor spread from the Cold Spring Harbor Meeting, I also learned that Susumu had already been bargaining with both *Cell* and *Nature* for quickest possible publication.

After Peter Newmark's call I rushed from my small office, shouting, 'Hey, everybody listen, would you believe it! CELL and NATURE want our immunoglobulin enhancer paper! They both called me and ASKED for it! This is MOUNT EVEREST! We're SUPERSTARS!' There was excited yelling, mutual shoulder-patting and, admittedly, a good portion of naive hubris. It didn't last for long. My wife Marianne, always providing loving support especially in tough times, is good at keeping my feet on the ground in moments of over-inflated self-confidence: 'Giants, you guys? Isn't this like giant prawns where even the biggest ones are not all that big?'

Remembering that Ben Lewin had given a fair treatment to our first enhancer paper (that had come, mind you, from a relatively unknown junior group), I stayed with *Cell*, and so in the end did Tonegawa (Banerji et al., 1983; Gillies et al., 1983). David Baltimore and Michael Neuberger also published on immunoglobulin enhancers, but their data were less complete (Queen and Baltimore, 1983) or appeared substantially later (Neuberger, 1983). Didier Picard, an undergraduate and graduate student in my lab, later found upstream sequences of the Ig lambda gene to inhibit transmission of the enhancer effect, and characterized the Ig kappa enhancer (Picard and Schaffner, 1983, 1984). He impressed me so much with an essentially perfect draft of his very first publication that I hesitated to put my name on it.

The enhancer trap, a Hershey heaven

In parallel to the IgH efforts, we developed an 'enhancer trap'. It consisted of an enhancer-less, linearized SV40 genome that was unable to grow in transfected monkey cells unless it picked up one that had enhancer activity from a mix of sonicated, co-transfected DNA fragments. Our assumption – quite daring – was that the host cell would do it all: namely, trim the transfected fragments, fuse them to the ends of SV40 to produce circular DNAs and then allow the right one to multiply. When it worked it was praised as an elegant technique, but at the time it was uncertain whether it would ever succeed. Julian and Christoph Gehring, a short-term postdoc, did the initial construct and gave it a first try, Jean continued, but to have ends meet (literally!) the project needed Frank. He delivered – on time (Weber et al., 1984). A follow-up collaboration with the lab of Bernhard Fleckenstein (Erlangen) led to the isolation of the very strong, ubiquitously active cytomegalovirus enhancer, which is now widely used by biotech companies for protein production in mammalian cells (Boshart et al., 1985). Incidentally, a completely different enhancer trap system was developed in *Drosophila* by Walter Gehring and colleagues in Basel (Bellen et al., 1989).

Using our enhancer trap, we isolated one enhancer after another. It was 'Hershey heaven'! [For one of the founders of molecular biology, Al Hershey, heaven was 'To have one experiment that works, and keep doing it all the time' or, in a more wordy version, to come to the lab every morning, to perform a significant experiment, and to find out that it worked (Stahl, 2000)]. Regarding publications we had a good harvest with enhancers from different virus classes, but hardly from cellular genes. A nice example of the latter was the incorporation of upstream segments of either human or mouse metal-inducible metallothionein genes – the resulting SV40 recombinants could only grow in host cells whose medium was supplemented with zinc (Serfling et al., 1985). The downside of this string of success was that I missed the first train to enhancer-binding proteins.

Clairvoyants and potential discoverers

Considering our talkative attitude and meandering working habits, it was sheer luck that we did not get scooped on the enhancers; nor were we seriously challenged for priority after publication. Amusing, rather than annoying, were the few wise men, typically outing themselves at the bar after a conference's evening session, for whom the enhancer effect was either 'logical', 'obvious', or who had 'foreseen it' (without leaving a record) or, even better, 'would have predicted it'.

Would the enhancer effect have been discovered anyway? Certainly! The story was bound to unfold, but most likely in smaller bits, step by step, rather than in one fell swoop. Harbingers included the mentioned studies in Pierre Chambon's and George Khoury's lab on the 72 bp repeats of SV40 (Benoist and Chambon, 1981; Gruss et al., 1981) as well as the bipolar 'modulator' DNA of Max Birnstiel and his super-PhD student Rudi Grosschedl (Grosschedl and Birnstiel, 1980b; see also Grosschedl and Birnstiel, 1982; Grosschedl et al., 1983). A later investigation in Giovanni Spinelli's lab identified typical enhancer properties in a subsegment of this modulator (Palla et al., 1994). Coming from a totally different angle, Mario Capecchi observed in cell transformation experiments a greatly increased number of colonies positive for thymidine kinase (TK) if the input DNA harbored, besides a viral TK

gene, also the SV40 origin of replication region. However, in an extrachromosomal state, TK expression was high even without SV40 DNA, strongly suggesting that the role of SV40 was to facilitate integration into the recipient cell's genome (Capecchi, 1980). On a similar track but using cellular DNA, Mike Botchan and Sue Conrad identified in the human genome multiple sequences with similarity to the SV40 origin region. When one of these was linked to a TK gene, it boosted TK expression in stably transformed cells (Conrad and Botchan, 1982).

A gene's major enhancer activity can be relatively close to the promoter region or even overlap with it, as in SV40 and some other viruses, the histone H2A, or metallothionein genes (but not the β-globin or immunoglobulin loci, see above). Given the still-strong influence of the bacterial promoter concept at that time, it is difficult to say how long it would have taken any of these other labs to realize that an enhancer-active segment could induce correct transcripts, also in an unrelated gene, independent of orientation and chromosomal integration, over long distances and even from a position downstream of the transcription unit.

Why activation over long distances?

With the publications on cellular enhancers in 1983 it became clear that enhancers were here to stay. And how they stayed! There were times when the human genome was thought to consist of 100,000 genes. While the gene number meanwhile has shrunk to about 25,000, the number of enhancers has gone on and on increasing, and often a given transcription unit is controlled by several enhancers. A classic study by Markus Noll and Werner Boll revealed a plethora of enhancers with different specificities, all of them associated with a single developmental regulator gene, the *Drosophila Pox neuro* locus (Boll and Noll, 2002). Nowadays, huge numbers of enhancers can be functionally identified with high-throughput techniques; a particularly ingenious one was developed by Alex Stark and colleagues (Arnold et al., 2013). The current estimate for mammals, mostly based on an analysis of enhancer-typical histone modifications, puts the number of enhancers at more than 300,000, perhaps as high as 1 million (ENCODE Project Consortium, 2012). And the maximal distance of activation was pushed farther and farther – some enhancers are located 1 million base pairs from the target promoter (for reviews, see Arnosti and Kulkarni, 2005; Visel et al., 2009; Levine, 2010; Bulger and Groudine, 2011; Spitz and Furlong, 2012; Levine et al., 2014; Morange, 2014; Shlyueva et al., 2014). Mammalian chromosomal domains span long DNA segments in the range of one megabase and enhancer-promoter interactions are usually confined to the same domain. Nevertheless – how can transcription be activated over distances of thousands, even hundreds of thousands of base pairs? It was already suspected early on that enhancers and promoters communicate by physical interaction, whereby the intervening DNA is looped out (Figure 3). Evidence supporting such a scenario has steadily increased (Müller-Storm et al., 1989; Su et al., 1991; Martin et al., 1996). In many cases, enhancer-promoter contacts are stabilized by cohesins, proteins that also play a role in chromosome pairing. How does each enhancer find its target? Proximity to one vs. anther promoter helps, but there are numerous exceptions, and open questions remain in spite of recent progress such as the finding that *Drosophila* 'housekeeping' genes specifically interact with an enhancer class of their own (Zabidi et al., 2014). One also may wonder, why remote control? Wouldn't it be simpler to have all the DNA elements for transcription control close to the transcription start? This is indeed the rule in simple eukaryotes such as yeast, and even in the fruit fly *Drosophila*, enhancers are less numerous (50,000–100,000) and less widely dispersed than in mammals (Kvon et al., 2014; in this latter study, the estimate was based on functional tests). I suspect that on the one hand, the selection pressure for economizing on genome size in short-generation-time organisms may contribute to this difference; on the other hand, many mammalian genes are expected to be controlled by tens of enhancers and it might be too difficult to pack all of them into a contiguous DNA stretch without them interfering with each other.

Enhanceosomes and the activation from close-by

Having worked for many years on sea urchin histone genes, SV40, and heavy metal-responsive genes I couldn't fail to notice that there are genes with strong enhancer activity close to, or even overlapping with, the promoter region, including the aforementioned genes for metallothioneins, histones of early cleavage stages, viral proteins in SV40 and also other viruses such as cytomegalovirus and retroviruses, heat shock proteins, and the anti-viral interferons. For the latter ones, the upstream enhancer region was described as a well-structured entity ('enhanceosome') with specific activating factors and 'architectural' proteins (Thanos and Maniatis, 1996). A

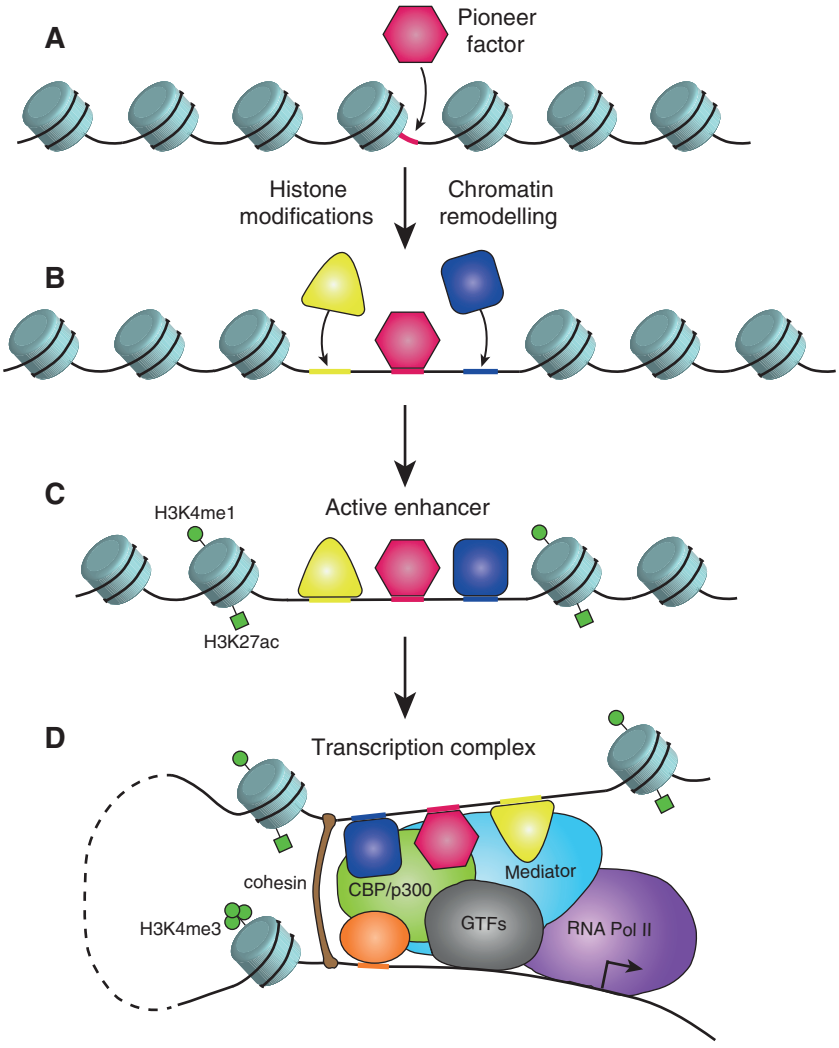

Figure 3: Activation of an enhancer.
(A) Schematic view of an inactive enhancer that is packaged into chromatin and poorly accessible for DNA-binding transcription factors. Histones in these nucleosomes carry posttranslational modifications characteristic for inactive, 'closed' chromatin (not indicated here). (B) Chromatin may be opened by a 'pioneer factor', a DNA binding transcription protein that is able to nevertheless occupy its binding site and will attract chromatin remodeling proteins and histone modifying enzymes. (As an alternative to a pioneer factor, a sufficient concentration of several DNA-binding transcription factors can cooperatively occupy an enhancer.) (C) Depletion of nucleosomes and introduction of enhancer-typical histone modifications in the neighborhood (H3K4me1, H3K27ac) render the enhancer accessible to further DNA-binding transcription factors (and cofactors such as the mediator complex, see below) as a prerequisite for its activity. The pioneer factor at this stage might even become dispensable/replaced by another transcription factor. (D) Scheme of enhancer-promoter interaction via DNA looping. The concerted action of enhancer-binding and promoter-binding proteins including a set of general transcription factors (GTFs), coactivators such as p300 (which has histone acetylase activity), the mediator multiprotein complex and cohesin (which can stabilize enhancer-promoter interaction), enables RNA polymerase II to initiate transcription. A promoter-typical histone modification (H3K4me3) is also indicated; see also recent reviews (Levine, 2010; Bulger and Groudine, 2011; Zaret and Carroll, 2011; Spitz and Furlong, 2012; Calo and Wysocka, 2013; Grünberg and Hahn, 2013; Levine et al., 2014; Shlyueva et al., 2014). Observations of transcriptional 'bursts' and intermittent periods of inactivity (with the 'off' intervals becoming shorter upon higher transcription activity) may be the result of on-off enhancer-promoter contacts and/or other forms of dynamic interactions (Amano et al., 2009; Suter et al., 2011).

common denominator of most of these genes is that they have to quickly jump into action, which might be more difficult with a remote enhancer, be it that looping takes more time, or that loops are less stable especially under stress. Under such conditions, splicing might also become a burden; metallothionein genes have short introns only, and genes for type I interferons, for a class of histones, and for the major heat shock proteins lack introns altogether.

Epigenetic marks, redundancy of function, synthetic enhancers

Typical cellular enhancers are several hundred bp long and represent an array of binding sites (6–12 bp each) for a multitude of DNA-binding transcription factors. Single, isolated binding sites out in genomic no-man's-land are usually unavailable for factor binding, because of the packaging of DNA into nucleosomes; only when sites are clustered do transcription factors have a chance to bind and prevail over nucleosomes, whereby a 'pioneer factor' might pave the way for others (Figure 3). The active state of enhancers is reinforced by 'active', or 'open' chromatin where nucleosomal histone proteins are specifically modified, notably by monomethylation of lysine 4 and acetylation of lysine 27 in histone H3 (H3K4me1 and H3K27ac, respectively). The acetylases and methylases responsible for these epigenetic modifications are brought to the enhancer region via direct or indirect binding to DNA-bound transcription factors. 'Open' chromatin is particularly sensitive to DNase treatment, a property that has long been exploited in the search for candidate enhancers.

Classical genetics dealt with the terms 'necessary but not sufficient' or 'necessary and sufficient'. For example, deletion of a protein-coding segment that results in loss of gene activity shows that the segment is necessary; however, to restore the function in a mutant organism, a complete gene has to be provided (necessary and sufficient). A surprising and seemingly paradoxical property of enhancers is that many of them are 'sufficient but not necessary'. For example, a virus with only half of its enhancer might still grow in cell culture (Schaffner et al., 1988); however, in a direct competition the wild type virus would win. Mike Levine and colleagues noted that in *Drosophila*, some regulators of development are under the control of two enhancers, whereby one of them was dubbed shadow enhancer. Each one of these enhancers can induce the same or a similar pattern of gene expression, but under stressful conditions such as elevated temperature, both enhancers are needed to ensure robust regulation (Perry et al., 2010). Thus what appears at first sight as a luxurious redundancy, upon closer investigation reveals itself as a feature of increased fitness under adverse conditions.

As the building blocks of enhancer activity are short DNA sequence motifs, synthetic, highly active enhancers can be constructed that consist of multiple binding sites for just one type of transcription factor (Gerster et al., 1987; Ondek et al., 1987; Schatt et al., 1988). Such monotonous enhancers do not occur in nature because they would not be compatible with a sophisticated regulation by different transcription factors and signaling pathways. Nevertheless, synthetic enhancers are useful when only one specific function is required: for example, multiple copies of binding sites for the transcription factor Gal4 are widely used for controlled transgene expression in *Drosophila* (Brand and Perrimon, 1993).

Of small enhancers and super-enhancers

Most viral genomes are subject to a strong size constraint and thus are more densely packed with genetic information than the typical chromosomes of eukaryotic cells. It is therefore to be expected that viruses also economize on the size of their transcription enhancers. Indeed the very small hepatitis B virus (genome size 3.2 kb) harbors an enhancer of some 170 bp that is even contained within the coding sequence for viral polymerase – quite a feat considering that two independent functions have to be preserved: enhancer activity and protein activity (Tognoni et al., 1985). In the somewhat larger polyomaviruses (genome of 5.2 kb), the enhancer is part of the non-coding control region and appears to be suboptimal for efficient transcription. When such 'archetype' viruses are adapted to cell culture or otherwise freed from the threat of the host's immune defense, variants appear that have undergone rearrangements in the enhancer, resulting in stronger early gene expression and more rapid virus growth (see also Figure 2A). The BK polyomavirus (BKPyV) is a paradigm of this phenomenon. BKPyV is widely distributed in human populations (prevalence about 80%) and typically persists as a commensal, symptomless virus. However, in immunocompromised individuals, virus variants of increased growth efficiency and pathogenicity often emerge. Their analysis reveals multiple rearrangements of the enhancer, typically duplications of some and deletions of other parts (Moens et al., 1995; Gosert et al., 2008); the most likely explanation is that the BKPyV archetypal enhancer has evolved to keep viral gene expression under careful control, such that the virus replicates just enough to be maintained within the host but remains 'under the radar' of the immune system. The longest, and also strongest, viral enhancers are found in cytomegaloviruses, large members of the herpesvirus family (Boshart et al., 1985; Dorsch-Häsler et al., 1985).

Rick Young and colleagues have noted that genes coding for master regulators of cell identity tend to be associated with 'super-enhancers', extended DNA segments that harbor several enhancers. Of the many enhancers that

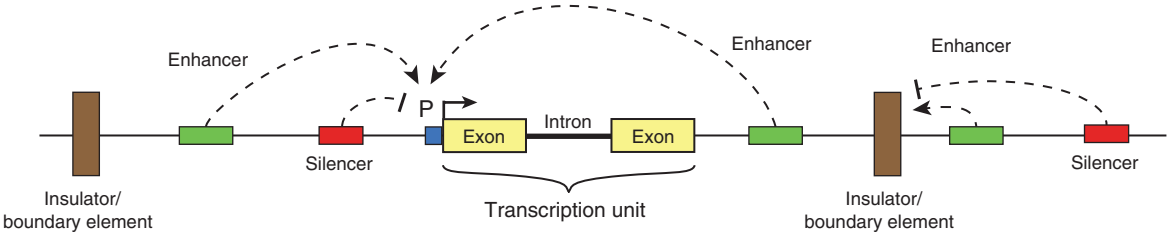

Figure 4: Landscape of *cis*-acting DNA regulatory sequences.
Depending on the cell type and signaling state, the promoter (P) of a transcription unit may be activated by one or the other enhancer, or kept in a repressed state by a silencer (Brand et al., 1985). Other enhancers or silencers (right-hand side) cannot influence activity, due to the shielding effect of insulator/boundary elements (Kellum and Schedl, 1991). Note that both Figures 3 and 4 represent simplified schemes. Depending on the cell type and the specific gene, both the DNA binding transcription factors and the composition of the multiprotein coactivator complexes can vary. Furthermore, insulators can bracket more than one transcription unit and multiple enhancers, and an enhancer may skip a proximal transcription unit to activate a more distal one. Additionally, enhancers and silencers are not always physically separated; binding of repressing factors or co-factors to an enhancer region can prevent its untimely activation. Enhancers typically work *in cis*, i.e., on the same DNA molecule as shown here, but in some cases, notably in the paired chromosomes of *Drosophila,* they can also act on the corresponding gene of the homologous chromosome. The latter phenomenon called transvection (Judd, 1988).

are active in a given cell, a minor subset is clustered into such super-enhancers (Whyte et al., 2013). The latter were defined by their large size, in the order of 20 kb; a high representation of binding sites for key regulators, such as the PU.1 transcription factor in B lymphocyte precursor cells; clusters of DNase I hypersensitivity sites; the enhancer-typical histone modifications, and an overrepresentation of bound 'mediator', a coactivator complex that facilitates recruitment of RNA polymerase II to the promoter. Extended enhancer regions that would qualify as super-enhancers were also identified by others. In addition to examples in *Drosophila* such as the mentioned *Pox neuro* locus (Boll and Noll, 2002), Frank Grosveld and colleagues found a 'locus control region' (LCR), a cluster of remote enhancers that regulates expression of the different β-type globin units (Grosveld et al., 1987; Talbot et al., 1989) and de Laat and Duboule (2013) described a 'global control region' (GCR) of multiple enhancers that regulates hand and digit formation by the HoxD gene cluster. In cancer cells, genes for oncogenic drivers such as c-Myc are also associated with super-enhancers (Loven et al., 2013). Generally, genes with super-enhancers are strongly expressed but have an 'Achilles heel': they are particularly sensitive to a reduction of the mediator complex and of BRD4, a bromodomain coactivator. Accordingly, therapeutic reduction of BRD4 and mediator appears to be a promising venue for anticancer therapy (Loven et al., 2013 and references therein).

Epilogue

Since 1981, a discovery made in a small DNA virus has developed into a universe of transcriptional gene regulation, advanced primarily by experiments in insects and mammals. Enhancers, in concert with other cis-active elements such as promoters, silencers (Brand et al., 1985) and boundary elements/insulators (Kellum and Schedl, 1991) (Figure 4), play a central role in normal and pathological processes – consistent with the notion that the majority of disease-associated human single-nucleotide-polymorphisms (SNPs) are located in the non-coding part of the genome (Rada-Iglesias, 2014). Enhancers can even be responsible for curious effects such as determining the blond hair color of northern Europeans (Guenther et al., 2014). Undoubtedly we know a lot – but how much don't we know? Even after more than three decades of study, important aspects of enhancer biology remain to be clarified, including the exact sequence requirements, the precise roles of the different transcription factors and cofactors, and the wiring of enhancers to their correct target promoters. Why did we not harvest a plethora of cellular enhancers in our SV40 trap experiments – is a typical enhancer not compact and active enough to fit into a space-constrained viral genome? As enhancers contribute to viral host range, could this reflect a passive defense mechanism of the organism to avoid enhancers being captured by viruses? Apart from all this I find it a mystery how a cell, especially a mammalian cell, manages to coordinate its countless enhancers, DNA loops, transcription factors and regulatory RNAs, not least because most of them may be expected to also produce some undesired off-target effects. What is more, we know little about how genes and especially their regulatory elements have governed the evolution from one type of body plan to another, with all the necessary physiological adaptations. Even with access to the complete genomes of several mammals, birds and reptiles, the maps of all enhancers, and tissue-specific

protein expression patterns, we would be at a complete loss to design a perfectly viable, close mimic of a dinosaur. (Or, to name a truly grand vision: a dog that closes the windows when a thunderstorm is approaching, instead of hiding under the sofa.)

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