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CRAcking the Code without Rosetta: Molecular Regulation of Calcium-Stimulated Gene Transcription after T Cell Activation

Mark Boothby

After a “Big Bang” (1, 2) begat an ever-expanding universe of identified genes (CD Ags, cytokines, and so on) and insight into the structure of these genes, a pressing need in immunology was to understand how changes in gene expression are regulated during immune responses. For this purpose, the promoter regulation model of Monod and Jacob (3, 4) and other lessons from the microbial world provided a compelling paradigm, that is, that sequence-specific DNA-binding proteins would provide an answer (5–7). Moreover, in the halcyon times of the 1980s, an organizing event for regulation of adaptive immunity was thought to be the activation of a T lymphocyte through engagement of its Ag receptor. Indeed, it became clear that a complex pattern of changes in mRNA levels followed T cell activation. Accordingly, an urgent challenge was to understand what DNA-binding proteins (direct regulators of gene expression, it was hoped) might be involved in controlling genes after Ag receptor stimulation and to determine how they would change their levels in the T cell nucleus after lymphocyte activation.

Unlike in bacteria and viruses, however, the analyses of promoter mutations in eukaryotic cells relied on generation and somewhat cumbersome molecular mutagenesis of promoters in reporter constructs. For mammalian systems, random and targeted mutagenesis of the germ line and screening to identify regulatory proteins were, at best, pipe dreams somewhere on the horizon, not the widespread realities they are today. The Crabtree laboratory’s publication of a Pillars article on this subject (8) was immediately recognizable as a major contribution and a big step toward the broader goal of using T cell activation as a model for understanding TCR-stimulated mechanisms controlling entire programs of gene expression. What was less clear at the outset was the breadth of the contribution in areas outside T cell biology and how central the protein identified as nuclear factor of activated T cells (NFAT) would become to our thinking about calcium-regulated gene transcription.

With necessity and passionate curiosity being the mothers of invention, one simple solution to this need to understand trans-acting regulators of gene expression was developed in the form of the EMSA (9). Thus, one could end label specific pieces of DNA, mix them with the complex solutions yielded by extracting nuclei for the nucleoplasm, and look for a change in migration after electrophoresis on non-denaturing gels. In the presence of nucleic acids designed to saturate associations of proteins binding to DNA, almost any loosely associated chromatin protein could be detected. Such assays screening for sequence-specific DNA-binding proteins (10) lent themselves readily to comparisons of multiple cell types and cells stimulated in various ways and analyzed across time courses. In addition, specificity controls using DNA containing the probe sequence or point mutations compared with the probe sequence and more refined “footprint” analyses (11) could be deployed to establish with confidence and good molecular detail the site(s) within a regulatory element with which the protein(s) in a nuclear extract interact. In the in vitro footprint analyses, one strand of a several hundred base pair promoter fragment was end labeled on one strand, and the positions of phosphodiester bonds relatively protected from scission were identified on DNA sequencing gels. These analyses enhanced the specificity and precision of localizing the sites recognized by proteins present in the nuclear extract. Simple comparisons of a resting and stimulated T cell line, Jurkat, and of this line to lines representative of other cell types, identified a rapidly induced protein that specifically recognized one of the functionally mapped Ag receptor-responsive cis elements in the IL-2 promoter. The mark of an important discovery is its influence on later research. What did this simple finding yield?

A first impact of this work was on understanding, at a level of molecular mechanisms, the ways by which immunosuppressant drugs could work. Induction of NFAT was soon found to be blocked by the drug cyclosporin A (12) and to be triggered by a calcium signaling pathway known to be central to T cell activation (13, 14). These two themes were linked by beautiful insights into the mechanism of action of the immunosuppressant cyclosporin A, showing that it acted in a complex with an endogenous cellular protein of entirely unrelated function to generate a unique molecular surface that inhibits the calcium-regulated serine-threonine phosphatase calcineurin (15). Thus, cyclosporin on its own, in the absence of cyclophilin A, is unable to inhibit the calcineurin-mediated dephosphorylation of NFAT to allow release of the transcription factor from phosphorylation-dependent retention in the
cytosol. Conversely, on their own neither cyclophilin nor its peptidyl prolyl isomerase activity regulates calcineurin or NFAT.

Another immediate impact of this *Pillsars* paper was that of identifying a high-priority target for molecular cloning. cDNAs encoding NFAT(s) turned out to be quite difficult to identify because the easiest methods that evolved during this era did not work, but in time, first one NFAT and then a family of NFAT proteins emerged (16, 17). These cloning efforts were the essential basis for gene targeting studies that firmly established the initial promise of NFAT as a TCR-induced regulator of immune function genes (18–21). Remarkably, though, these lines of work led to two further paradigm-shifting discoveries. First, a surprising finding was that a compound loss-of-function state for two members of the NFAT gene family led to unchecked inflammation and T cell hyperactivation (18, 22, 23). How could this be? Ultimately, the paradox could be interpreted in the context of the rise of the suppressor cell, that is, dominant tolerance effected by natural regulatory T cells (Tregs). Second, NFAT and the relationship of its activation to SOCE (store-operated calcium entry) were brilliantly exploited as part of the groundbreaking work identifying Orai1 (24) as an essential component of SOCE (along with STIM1 and STIM2) and leading to identification of an essential role for this process in development of natural Tregs (25, 26).

During the era when the universe of transcription factors was young and undergoing explosive expansion, two themes were woven into investigations. The first was that transcriptional function is built from the combinatorial interaction of several proteins, for instance, at adjacent cis-acting elements. In this respect, a germinal contribution was the identification of a crucial interaction between NFAT and dimeric proteins of an AP-1 family of transcription factors. This insight was a progenitor of a fundamental advance in thinking about unresponsiveness of conventional T cells, especially helper-type CD4 T lymphocytes (27). In particular, clonal anergy (28) can now be viewed as a consequence of nuclear NFAT, arising from sustained low-level calcium signaling in tolerized lymphocytes, when it lacks an AP-1 partner and therefore drives expression of a suite of genes differing from those of normal T cell activation (29). This paradigm, together with findings about the role of NFAT in suppressive Treg development, is valuable in the context of down sides to the chronic use of cyclosporin A, that is, its enhancement of a chronic vasculopathic effect.

A second theme integral to investigations of DNA-binding transcription factors is that a particular mobility shift complex, or eukaryotic transcription factor, must necessarily regulate far more genes than the one at which its function was first implicated. Thus, the identification of NFAT as an immediate-early factor induced by T cell activation to trans-activate IL-2 gene transcription was followed by a stream of evidence showing that NFAT regulates scores of other genes in the activated T cell, including those encoding effector cytokines such as IL-4 and IFN-γ. Inasmuch as the effect on immunity of chronically suppressing IL-2 production became a more complex picture, these diverse effects on effectors of immunity or allograft rejection add in important ways to the understanding of how calcineurin phosphatase inhibition prolongs graft survival.

There are certain periods when an explosion of foundational insights in an area of science can seem like the efflorescence that moved physics in one memorable era or painting in an-
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