RESEARCH NEWS AND VIEWS

Debugging expression screening

Philip L. Felgner and Xiaowu Liang

Access to the complete genome sequence information for an ever-growing list of organisms has ushered in a new era in biology and biotechnology. Where we once sought genes for functions, we now increasingly seek functions for genes. The limiting factor in many "functional genomic" strategies is the sheer volume of manipulations needed to screen systematically large numbers of gene products for a particular activity, and this has led to the introduction of robotics and other high-throughput technologies into the biology laboratory. The advent of PCR freed us from the need to rely on microorganisms to produce useable quantities of DNA, but recombinant-DNA technology has until recently still required the generation and growth of individual clones in bacteria. In this issue, Sykes and Johnston[1] describe a simple but elegant new procedure that has the potential to erode further the predominance of Escherichia coli as the preferred medium of recombinant-DNA manipulation.

The new approach is based on the surprising finding that amplified PCR fragments could be rendered transcriptionally active by simply hybridizing them to active promoter and terminator sequences. PCR primers were chosen so that their termini would be complementary to two additional PCR fragments encoding a promoter sequence and a terminator. When the complementary promoter, coding, and terminator fragments were mixed, they spontaneously hybridized to form linear-expression elements (LEE) that could be transfected into cultured cells or injected into animals, leading to high-level expression of the coding sequence. Significantly, when antigens from the genome of the important human pathogen, Mycobacterium tuberculosis, were used as coding sequences and the transcriptionally active LEE was administered intradermally by gene gun or injected intramuscularly, the injected animals developed antibodies against the tuberculosis antigen. Sykes and Johnston suggest that one of the uses for LEE will be to identify effective antigens for DNA vaccines.

Since its initial description[2–5], DNA vaccination has moved far beyond the level of a novel observation and is now a steadily maturing segment of immunology research and clinical vaccine development in animals and in humans. Over 800 scientific publications have appeared[6], dozens of effective DNA vaccines have been demonstrated in animals, and successful immunizations have also been conducted in man[7]. These results have established that when plasmids encoding heterologous antigens are injected into animals, the gene products generated in vivo stimulate antigen specific humoral and cell mediated immune responses that are often sufficient to protect animals from a live pathogen challenge.

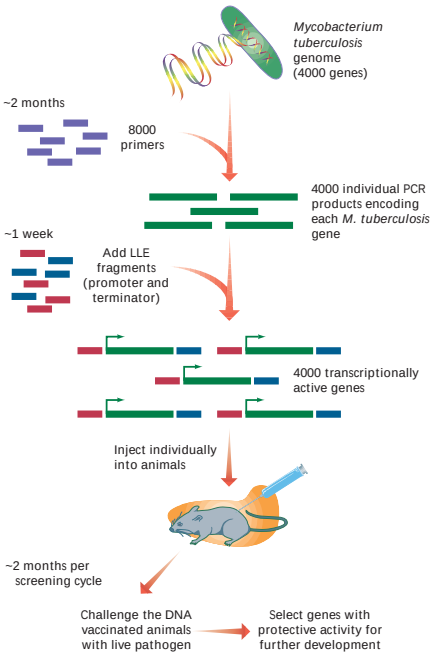

Figure 1. The LEE approach to expression library immunization. In this example, the 4000 genes of M. tuberculosis are individually amplified from the genome by PCR. Each PCR product is annealed to LLE fragments encoding promoter and terminator sequences, and is injected either individually or as part of a small pool into animals.

One of the more difficult tasks in developing a vaccine is the identification of the particular antigen that will stimulate the most effective immune response against the pathogen, a task that becomes even more complex as the size of the genome grows. A comprehensive way to accomplish this would be to obtain each of the structural, metabolic, and regulatory antigens to the pathogen and to test their protective immunity individually or as mixtures in the vaccine. To develop an influenza A DNA vaccine, for example, 11 expression vectors encoding each of the 11 open reading frames from the virus genome can be separately prepared. The purified plasmids can then be injected, either individually or as mixtures, into laboratory animals; immune responses against the expressed antigens can then be monitored and the animals challenged with live virus. An effective DNA vaccine candidate would be one that contains a single plasmid or mixture of plasmids conferring protection against the live-virus challenge. However, the utility of this approach decreases as the size of the organism increases: the genomes of *Borrelia burgdorferi* (Lyme disease), *Chlamydia* spp., *Helicobacter pylori*, and *M. tuberculosis* encode 850, 900, 1,600, and 4,000 genes, respectively. The genome of Plasmodium falciparum, the parasite responsible for malaria, is predicted to encode more than 6,000 proteins, each of which is a potential antigen for a DNA vaccine. The time and expense of individually cloning thousands of genes from these larger organisms is prohibitive using current methodologies.

For several years the Johnston lab has been interested in tackling this challenging problem. They discovered that immune responses against a specific antigen encoded on a plasmid could be obtained even when the plasmid was a minor component in a large library of plasmids encoding many different antigens[8]. This knowledge led to their description of "expression library immunization" (ELI), which could be used in principle to identify and isolate protective antigens from large expression libraries encoding many different antigens from bacteria or parasites. They proposed that if larger libraries could be segregated into smaller "sibling" libraries with fewer members, eventually immunologically active sequences could be isolated and identified. While this approach was feasible in principle, it required the analysis of purified plasmids from a large number of different libraries and individual clones.

In contrast, the LEE approach has the potential to greatly accelerate the rate at which effective DNA vaccine antigens from parasites and bacteria can be identified, without the limitations of the ELI method. Using mycobacterium tuberculosis for example (see Fig. 1), 8,000 oligonucleotide primers

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suitable for amplifying all 4,000 TB genes can be obtained from a commercial supplier in about 2 months time. Within about another month all 4,000 TB genes could be amplified and organized into about forty 96-well plates, and using the LEE approach, the promoter and terminator sequences could be added to the amplified fragments. These transcriptionally active LEE fragments could be organized into 40 pools containing about 100 fragments, and each pool could be evaluated for in vivo immunologic activity. Active pools could be further segregated into smaller pools or the fragments could be individually evaluated for in vivo activity. In this way the immunologically active antigens suitable for a DNA vaccine could be comprehensively identified.

In addition to pointing the way toward an improved method for identifying immunologically active antigens in complex organisms, the LEE approach should find broader uses as a genomics tool to help elucidate the function of undefined genes. It could be used to produce antibodies against proteins even before they have been cloned and expressed. And finally, chemically modified linear-expression elements may eventually replace plasmids in synthetic gene delivery systems for many more gene therapy applications[9,10].

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Transgene escape and transplastomics

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Genetically modified (GM) food is big news at the moment, particularly in Europe. Hysteria seems to have gripped the British press (from the low-brow tabloids to the highbrow broadsheets) in a furor of at least the magnitude of *Salmonella* in eggs and BSE in beef (food scares seem to be a special favorite of Fleet Street). Press releases appear weekly with descriptions of the latest anti-GM crop activities of groups like Greenpeace, who have, for instance, deposited 4 tons of GM soybeans on Tony Blair's doorstep, and filed a lawsuit against the EPA for approving transgenic plants carrying the *Bacillus thuringiensis* toxin (see http://www.greenpeace.org for details). The UK government is reappraising its stance on commercial growing of GM crops, and Monsanto was fined in Lincolnshire, England for failing to conduct proper field trials. Clearly, the use of transgenic technology—and the perceived threat of uncontrolled transgene spread—is a hot, organically produced, nontransgenic potato.

In this issue, Scott and Wilkinson[1] assess the probability of pollen-mediated movement of transgenes from transplastomic (rather than nuclear transgenic) *Brassica napus* to its wild relative *Brassica rapa*. Proving that transgene escape from transplastomic crops poses a negligible risk would do much to support the use of this technology for containment of transgenes. But it's important to remember that for any transgene to spread (nuclear or plastomic), there must be successful hybrid formation between a sexually compatible crop plant and recipient species. The two species must flower at the same time, share the same insect pollinator (if insect-pollinated), and be close enough in space to allow for the transfer of viable pollen[2]. Thus, the transfer of transgenes will depend on the sexual fertility of the hybrid progeny, their vigor and sexual fertility in subsequent generations, and the selection pressure on the host of the resident transgene[2,3].

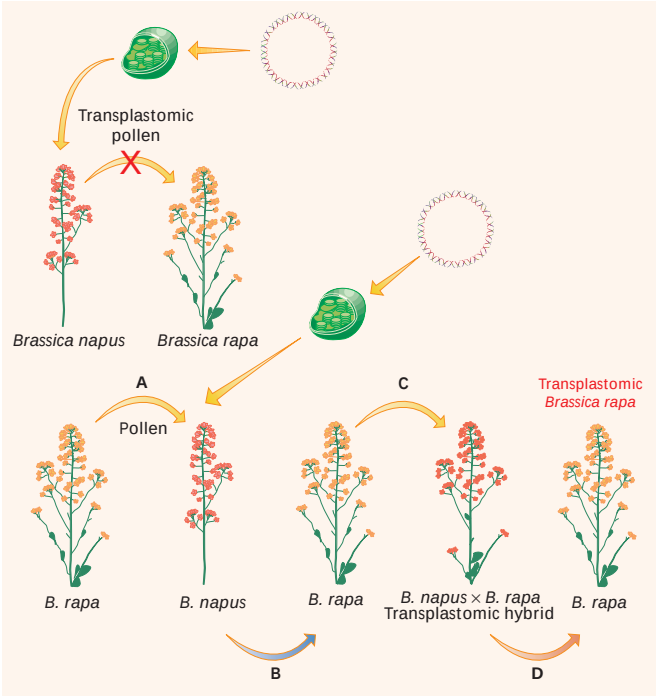

Figure. (A) Transplastomic oilseed rape (*Brassica napus*) transgenes will not flow into related weeds (e.g., *Brassica rapa*) through pollen. (B–D) If transplastomic oilseed rape served as the female parent, then transgenes could be introgressed into the weed *B. rapa*. Transplastomic oilseed rape plants might be rare in a wild *B. rapa* population and might be pollinated by wild *B. rapa* (A). Some of the progeny would be transplastomic hybrids (B). After a single backcross of the transplastomic hybrids with wild *B. rapa* pollen (C), some of the progeny would be functional transplastomic *B. rapa* (D).

Generating transgenic chloroplasts with biolistics is still difficult, as is selecting a pure population of transformed chloroplasts. In the 9 years since the first transplastomic higher plant was generated[4], stable biolistic chloroplast transformation in plants has been achieved in only one species: tobacco[5,6]. A major drawback with transforming the chloroplasts of agronomically important crops is that gramineous embryogenic plant cultures contain proplastids that are smaller than the projectiles used for biolistic plant transformation[7]. So at present, it seems unlikely that the success rate of generating transplastomic crops will ever approach that of nuclear transformation.

But if we assume that transplastomic oilseed rape is possible to produce, will use of this technology translate into transgene containment? Scott and Wilkinson describe an interesting scenario that addresses this issue. First, consider a feral wild-type population (*B. rapa*) that is contaminated

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