Perspectives in Diabetes

Transgenic Mouse Models of Type I Diabetes

MYRA A. LIPES AND GEORGE S. EISENBARTH

Transgenic mouse technology has gained recognition as an important tool for examining many fundamental biological questions in vivo. Recently, transgenic mouse techniques have been applied to the study of type I (insulin-dependent) diabetes. These studies have been particularly informative in elucidating *1*) mechanisms whereby immune tolerance is maintained to antigens on rare specialized cells such as the pancreatic β-cell, *2*) disease susceptibility and resistance genes, and *3*) potentially important immune effector mechanisms. In this article, we discuss these studies, their impact on understanding of the pathogenesis of type I diabetes, and the potential of the transgenic mouse approach for future research. *Diabetes* 39:879–84, 1990

The ability to introduce functional genes into the germ line of mammals has rapidly become a powerful tool in biological research. Through the use of this technique, new insights have been gained into understanding complex biological processes such as developmental gene regulation, the role of oncogenes in tumor formation, and the function and organization of the immune system. Furthermore, the technique offers the potential to develop animal model systems for human disease. If a putative disease-causing gene is known, the consequences of its expression can be studied in the context of the growth and development of the whole animal, an endeavor not satisfactorily achieved in cell culture. Recently, transgenic mouse techniques have been applied to type I (insulin-dependent) diabetes. What contributions have they made to further understanding of the disease pathogenesis? In this discussion, we analyze some of these current studies in the context of several key questions in the field of the immunology of type I diabetes. What triggers the disease? What genes underlie disease susceptibility? What molecules mediate pancreatic β-cell destruction? Before addressing these questions, however, we outline the basic strategy for making transgenic mice (Fig. 1). For further details, several excellent comprehensive reviews are available (1–3).

In general, microinjection of cloned DNA directly into the pronucleus of a fertilized egg is the most extensively and successfully used method for generating transgenic mice (1). DNA is introduced at the one-cell stage, before cleavage of the fertilized egg. The DNA integrates at one, presumably random, site in the genome. When more than one copy integrates, as is usually the case (up to several hundred copies can be integrated), they are organized in a tandem head-to-tail fashion, most likely by the process of homologous recombination. The microinjected eggs are then implanted into the oviducts of pseudopregnant foster mothers. After weaning, the progeny are "tailed," and DNA is prepared for Southern blot, dot blot, or more recently, analysis with the polymerase chain reaction technique (which can be performed on as little as a drop of blood; 4) to determine whether the transgene is present. Each transgene-positive (founder) mouse thus determined can be mated with a non-transgenic mouse to establish separate transgenic lineages within the colony. The transgene is usually inherited as a simple Mendelian trait. The developmental timing and level of expression of the transgene can vary greatly from one founder to another and are thought to depend primarily on the chromosomal insertion site of the transgene rather than gene copy number (1). Expression within each transgenic lineage is uniform, thus allowing for large-scale reproducible analysis of the functional consequences of foreign gene expression.

WHAT ARE THE TRIGGERS?

For nearly 100 years, immunologists have pondered how the immune system is capable of distinguishing between "non-self" and "self"; it is capable of responding to a seemingly

From the Immunology Section, Joslin Diabetes Center; Department of Medicine, Harvard Medical School; Brigham and Women's Hospital; and New England Deaconess Hospital, Boston, Massachusetts.

Address correspondence and reprint requests to Myra A. Lipes, MD, Immunology Section, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215.

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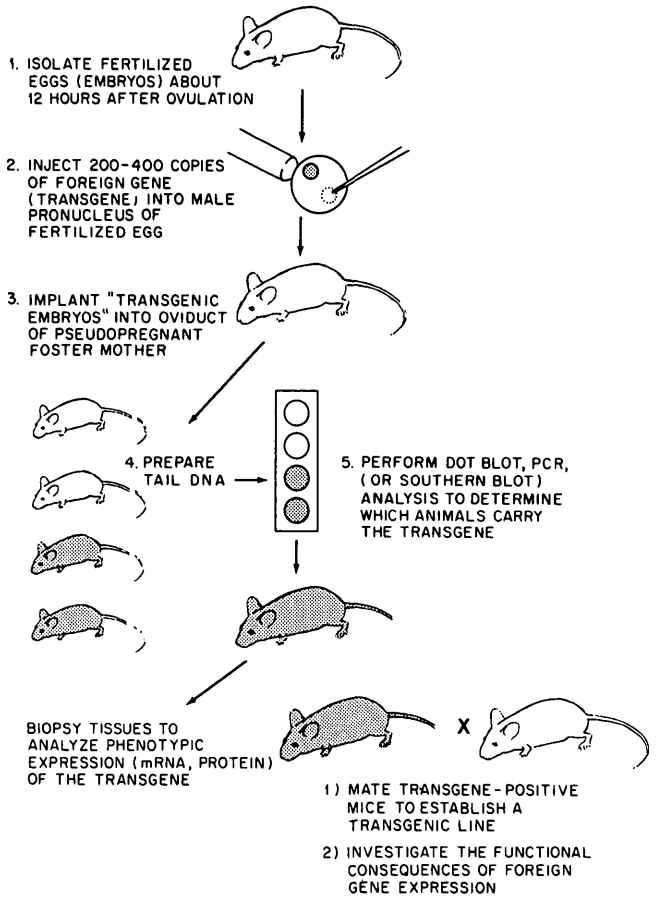

FIG. 1. Steps used in making transgenic mice.

infinite spectrum of foreign substances, but it is specifically unresponsive (tolerant) to self-molecules. Recognition of the former greatly benefits the host, protecting against infection (immunity), whereas failure to recognize the latter results in a deleterious attack against the animal's own tissues (autoimmunity). Some degree of autoantibody production, however, is compatible with health, as is some degree of anti-self T-lymphocyte reactivity. Despite the potential of individuals to mount an autoimmune response, autoimmune disease is a rare event. What has been learned about how immune tolerance is normally established and the mechanisms by which tolerance breaks down in type I diabetes?

It is unfortunately apparent that probably no single simple event represents "the" basis of immunologic tolerance (5). Normally, cells of the immune system pass through the thymus during development, and by the process of clonal deletion, lymphocytes bearing receptors capable of recognizing self-antigens are eliminated or inactivated. Clonal deletion of anti-self lymphocytes is a major, but not the sole, mechanism for generating tolerance; in athymic animals, self-reactive T lymphocytes do not get eliminated, but these animals do not routinely develop autoimmune disease (6,7). Furthermore, tolerance to many antigens present in small quantities on specialized cells located peripheral to immunologic organs must be explained.

In classic studies of immune tolerance, an investigator would artificially create a state of tolerance by administering a foreign antigen in a nonimmunogenic form and then readminister it in an immunogenic form. This model situation bears little resemblance to the real-life situation in which authentic self-antigen exists in the body from an early stage of embryonic development and is maintained at a constant concentration over long periods (5). This can be readily achieved by the whole-animal transfection with antigen genes of interest in transgenic mice, in which the introduced genes are expressed and treated as self-molecules. Furthermore, the identification of tissue-specific promoters in genes such as insulin has enabled investigators to target gene expression exclusively to defined tissues of interest, e.g., the β-cell.

However, the autoantigen(s) of primary importance in the activation of immune system in type I diabetes has yet to be identified. Defining the loss of self-tolerance in the absence of knowledge of authentic autoantigens has forced investigators to study tolerance to other antigens. One of the earliest and most penetrating transgenic models relevant to peripheral tolerance induction and to type I diabetes was developed by Adams et al. (8). In their study, a fusion gene containing the rat II insulin promotor (RIP) and the protein coding region for a nuclear oncogene, the SV40 large T antigen (Tag), was introduced into the germ line of B6D2 (C57BL/6J x DBA/2J) hybrid mice (9). Several lineages of RIP-Tag mice were then examined. Probably because of random chromosomal integration events, some lineages expressed the T-antigen transgene early in gestation and at high levels, whereas other lines of mice, characterized by late-onset expression, did not express the T antigen until 2–3 mo of age, and expression was at lower levels. This correlated well with tolerance induction. Mice that expressed the transgene early were tolerant. In contrast, the delayed expression of the transgene resulted in the production of autoantibodies against the T-antigen protein and the development of lymphocytic infiltrates in the pancreatic islets. Diabetes, however, did not develop. Interestingly, some, but not all, mice in the late-onset group developed this autoimmune response, and this was found to closely correlate with the MHC haplotype of the mice (10), reminiscent of the heritable susceptibility of type I diabetes in humans and animal models (see below). The autoimmune phenotype of this transgenic model thus hinged on the inability of these transgenic lines to express the viral antigen early in ontogeny. The results of these studies raise the possibility that, if β-cell injury (virus? environmental insult?) were to result in the late expression of a β-cell target autoantigen normally sequestered in the cell or cell membrane, this could likewise trigger anti-islet autoimmunity in genetically susceptible hosts.

Other mechanisms exist, however, to prevent autoimmunity from occurring. For example, unlike B lymphocytes, which recognize intact antigen free in solution, T lymphocytes "see" antigen in close association with the MHC class I (for most $CD8^+$ T lymphocytes) and class II (for most $CD4^+$ T lymphocytes) molecules. This corecognition is termed *MHC restriction*; T lymphocytes are blind to antigen alone. Furthermore, the influx of accessory factors (including cytokines such as interleukin 1 [IL-1]) secreted by the antigen-presenting cells is an obligatory requirement for the activation of T lymphocytes (11).

MHC class I molecules are ubiquitously expressed at low

levels by all nucleated cells, whereas MHC class II molecules normally have a more restricted tissue distribution and are expressed primarily on thymic epithelium and bone marrow–derived cells such as B lymphocytes and cells of the macrophage/dendritic cell lineage. The observation that expression of MHC class II molecules could be induced on thyroid epithelium by treatment with interferon-γ (IFN-γ) and that these cells were then capable of presenting foreign antigen to antigen-specific T-lymphocyte clones (12), along with the finding that class II MHC expression correlated with the antigen-presenting function in transfected fibroblasts (13), led to the speculation that perhaps any cell induced to express class II molecules might also acquire antigen-presenting function. For the β-cell, it was hypothesized that aberrant expression of MHC molecules, induced by viral injury and subsequent local cytokine production, could itself enable the β-cell to function as an antigen-presenting cell, present autoantigens in a novel way, stimulate T lymphocytes, and thus trigger autoimmunity (14). Interest in the potential relevance of this hypothesis to type I diabetes was intensified by the finding of class II expression on the β-cells of patients with insulitis and newly diagnosed type I diabetes (15). However, an equally plausible alternative view was that class II expression was a secondary effect of the inflammatory infiltrate and local cytokine production (16).

To test whether enhanced expression of class I or de novo expression of class II genes was sufficient to cause lymphocytic invasion, autoimmune destruction of β-cells, and diabetes, several groups made transgenic mice in which expression of the class I molecule H-2K^b (17) and the class II molecules I-E^b (18) and I-A^d (19) was targeted to the β-cells via the rat insulin promoter. As expected, this resulted in high-level expression of MHC molecules on β-cells with little expression elsewhere. In all three cases, the transgenic mice developed early-onset severe diabetes. Unfortunately, there was no evidence of immune involvement in any of these cases. In some cases (class I and class II I-A), the islets appeared disorganized, with degranulated pleiomorphic β-cells. Little or no insulin was detectable in these transgenic mice by immunohistochemistry, with significant reduction in insulin secretion detectable as early as day 15 of fetal life (17). The most likely explanation of these findings was that the overexpression of MHC molecules disrupted insulin secretion by β-cells, perhaps by binding to insulin and preventing its delivery to secretory granules (20). In the light of these concerns, Bohme et al. (21) constructed transgenic mice that, by virtue of a cDNA transgene construct, expressed much lower levels of MHC class II molecules, comparable to levels found on resting B lymphocytes. Again, there was no evidence of immune involvement in these mice, and diabetes did not develop.

One puzzling observation in the above-described studies was that the immune system of the transgenic mice did not appear to reject (or even recognize) the foreign MHC transgene products. Why did immune tolerance occur instead of rejection? One possibility was that, despite the expression of MHC antigen on β-cells, the immune system may have failed to "see" the foreign transgene products because of inefficient antigen presentation. Tremendous variability was previously noted in the efficiency of antigen presentation even among "professional" antigen-presenting cells: macrophages appeared to be as much as 10,000-fold more potent than resting B lymphocytes in presenting antigen (21,22). To examine this issue, Markmann et al. (23) grafted I-E–expressing fetal pancreas from the class II I-E–expressing transgenic animals of Lo et al. (18) into naive I-E–negative adult recipients. The transgenic islets were accepted without evidence of rejection. However, when conventional antigen-presenting cells (spleen cells) were added, complete destruction of the transgenic grafts occurred rapidly. These in vivo experiments were confirmed by in vitro studies, which demonstrated that I-E–expressing islets were unable to present peptide antigen to T-lymphocyte clones, but this could be rapidly reversed with the addition of spleen cells. One possible explanation for the need for conventional antigen-presenting cells is that they are a known source of cytokines and other costimulatory signals. The need for appropriate second signals for antigen presentation by the MHC-expressing β-cell was further emphasized by the studies of Morahan et al. (24) in which unresponsiveness to the foreign H-2K^b transgene product could be reversed in vitro by providing recombinant IL-2 (24). These studies confirm previous demonstrations that T-cell receptor triggering in the absence of the appropriate second signals could lead to a nonresponsive tolerized state (25).

In conclusion, enhanced class I or de novo class II antigen expression is unlikely to be solely responsible for the initiation of autoimmunity. Although these transgenic studies provide a novel framework for understanding how autoimmunity may normally be prevented, the nature of the "initial lesion" remains a mystery.

WHICH GENES MEDIATE DISEASE SUSCEPTIBILITY AND RESISTANCE?

The genetics of type I diabetes in humans and rodent models is complex and poorly understood. In the NOD mouse, at least three recessive genes on independent chromosomes are involved in disease susceptibility. The best-studied gene is linked to the MHC region on chromosome 17. Overt diabetes depends on homozygosity at this locus. In view of this and the strong MHC associations seen in human type I diabetes, there has been tremendous interest in defining the precise MHC loci contributing to disease susceptibility in both humans and in rodent models.

The class II molecules are logical candidates for playing key roles in disease suceptibility because of their known regulatory role in the immune response. Clear-cut associations between MHC class II gene expression and immune responsiveness was originally shown by studies in which mice of different MHC haplotypes were found to differ markedly in their ability to respond to different antigens (26). Individual differences in MHC class II molecules are known to influence immune regulation at several levels.

1. The differences determine whether peptide antigen can be presented. Most proteins have only a few major processed peptides that are capable of appropriately associating with the MHC configuration of a given animal. Therefore, the possibility exists for nonrecognition of a particular protein if none of the processed peptides of a given molecule properly fit the particular MHC complex of the animal. This has recently been demonstrated in human hep-

atitis B vaccine recipients, in whom nonresponse was associated with certain MHC haplotypes (27).

2. The MHC molecule may work at a more quantitative level in influencing the magnitude of the immune response. As MHC haplotypes influence the nature of the peptide antigen presented (28), they can influence the strength of T-cell receptor–peptide antigen–MHC interaction and modulate the secretion of costimulatory signals/cytokines by both antigen-presenting cells and T lymphocytes.

3. MHC molecules may play a critical role in shaping the T-cell–receptor repertoire. MHC molecules are expressed in the thymus, where T-lymphocyte tolerance occurs by two processes: first by positive selection, in which T lymphocytes are only allowed to mature if their receptors recognize self–MHC molecules, and then by negative selection, in which T lymphocytes bearing receptors capable of recognizing self-antigens are deleted or inactivated (29,30).

4. MHC molecules may interact with a self-peptide to generate a distinct population of suppressor T lymphocytes that downregulate the immune response. Direct evidence for defects in this pathway in the pathogenesis of autoimmune disease, however, is lacking.

It was therefore of great interest to find that the NOD mouse possessed a unique MHC class II molecule: the I-A region (corresponding to DQ in humans) did not react with standard monoclonal antibodies, and the I-E region (the murine DR counterpart) was not expressed (31). Absence of I-E expression, however, is not unique to NOD mice and is shared by all mice of the *H-2*^{s} and *H-2*^{b} haplotypes (NOD *H-2K*^{d} and *D*^{b}) and by ~20% of wild mice. This defect has been attributed to a deletion in the promoter region and the first exon on the E_α gene; although the E_β gene product is produced in normal levels in the cytoplasm, in the absence of E_α, it is not expressed on the cell surface (32).

To examine whether there was a relationship between MHC I-E expression and immune dysfunction in NOD mice, Nishimoto et al. (33) mated NOD mice with C57BL/6 mice that were transgenic for the E_α^d molecule, which was expressed under its own transcriptional regulatory elements. As expected, all of the F_1 progeny expressed functional I-$E^{d/b}$ molecules. These mice were then backcrossed to NOD mice, and the I-E–positive and I-E–negative mice were examined. Interestingly, no insulitis developed in NOD mice in the presence of an intact I-E molecule. These remarkable findings have generated much speculation about possible mechanisms whereby I-E mediates its protective effect in NOD mice. Although it has been postulated that this effect may be mediated through the deletion of putative autoreactive Vβ families (e.g., Vβ5; 34), we recently found that, in T-cell–receptor β-chain transgenic NOD mice, autoimmunity developed despite depletion of these and other Vβ families (35).

In terms of evaluating the role of specific disease-susceptibility loci, much attention has been focused on characterizing the unique I-A β-chain of the NOD mouse. The I-A α-chain sequence is unremarkable (it is identical to the A α-chain in Balb/c mice; 36), and as mentioned above, I-E is not expressed. A major issue is whether disease susceptibility is also determined by class II genes in the I-Aβ complex or by different, closely linked genes and how the putative deleterious genes could contribute to disease pathogenesis. The hypothesis that susceptibility is determined by absence of Asp in position 57 in humans (in the DQ β-chain) and NOD mice (in the corresponding I-A β-chain) may be an oversimplification (37) in that several exceptions have emerged: *1*) type I diabetes in Japanese patients is associated with the DQβ3.3 haplotype, which has Asp at position 57 (38); *2*) BB rats, like NOD mice, have a Ser in position 57, but this residue is also found in diabetes-resistant strains (39); *3*) expression of the DR homologue I-E in transgenic NOD mice prevents insulitis (33); and *4*) no recombinants within the *H-2* region to precisely define relevant genes have been bred and analyzed. Several MHC alleles and potentially non-MHC–linked genes probably contribute to disease susceptibility. One way of testing with transgenic techniques whether position 57 is a major factor in determining disease susceptibility or resistance is to introduce into NOD mice an $A\beta^{NOD}$ gene in which the β-chain sequence has been mutated by site-directed mutagenesis so that position 57 is Asp rather than Ser. Such studies are under way in several laboratories.

WHAT ARE IMMUNE EFFECTOR MECHANISMS?

Although it is unclear what initiates the autoimmune process in type I diabetes, there is now much evidence that the effector phase of the immune response, by which we mean the mechanisms leading to functional and/or structural damage of the β-cell, is T lymphocyte dependent. Overt disease can be adoptively transferred with purified T lymphocytes (40,41) and more recently with antigen-specific T-lymphocyte clones (34) from diabetic NOD mice, and disease can also be prevented in NOD mice with therapies directed against T-cell subsets (42) or by neonatal thymectomy (43). Although T lymphocytes are essential for the full expression of diabetes, it is unclear whether their primary importance resides in their ability, by virtue of their specific receptors, to mediate cell-to-cell contact with target autoantigens or to secrete an armament of highly potent chemical mediators (lymphokines). Support for the latter as a potential mechanism for T-lymphocyte damage initially came from work on BB rats, in which disease could be induced in diabetes-resistant strains by the supernatants from concanavalin A–activated spleen cells, some from nondiabetic donors (44).

The consequences of sustained IFN-γ expression in the islets were examined in a transgenic mouse model in which expression was targeted to the β-cell by the rat insulin promoter (17). IFN-γ is a product of activated T lymphocytes and natural killer cells and is normally produced by the host in response to viral and bacterial infections. One of the major functions of IFN-γ is to recruit and activate macrophages, which can become cytolytic and capable of producing free radicals such as the superoxide anion O_2^-, and cytokines such as IL-1 and tumor necrosis factor (TNF). In vitro studies by Okamoto (45) and Bendtzen et al. (46) have suggested that both these cytokines can be directly and selectively toxic to the β-cell. Another function attributed to IFN-γ is the ability to upregulate MHC class II gene expression on various lymphoid and nonlymphoid cells in vitro and thus potentiate antigen presentation and immune recognition. There have been no published reports demonstrating that IFN-γ (or TNF) is actually made in physiologically relevant concentrations

in the insulitis lesion of NOD mice, but such measurements are difficult to obtain.

Studies in transgenic mice in which sustained IFN-γ expression was targeted to the islets revealed that an intense inflammatory infiltrate consisting of lymphocytes and macrophages developed early in neonatal life and involved the entire pancreas (19). In one of the three lineages, diabetes developed at an early age (6 wk). These studies demonstrate that IFN-γ, either itself or indirectly through its ability to recruit and activate other cells, is sufficient to initiate an inflammatory response and mediate β-cell destruction. It remains to be proved whether the process selectively involved the β-cell or, more important, that it was the consequence of an autoimmune response, but the destruction of transplanted islet tissue in this model indicates that IFN-γ–mediated activation of the immune response can target destruction of normal islets (N. Sarvetnick, unpublished observations). Undoubtedly, similar transgenic mice expressing IL-1 and/or TNF will also be interesting for comparison.

Our own work with transgenic mice has focused on the nature of T-cell receptor recognition in the NOD mouse (35,47). We introduced a functionally rearranged T-lymphocyte–receptor β-chain gene from a T-lymphocyte hybridoma with foreign antigen specificity (chicken ovalbumin) and MHC restriction (I-A^d) into the NOD germ line. This resulted in a high-level expression (97% of peripheral T lymphocytes by fluorescence-activated cell sorter analysis) of the transgene with a concomitant dramatic depletion (<0.1%) of the endogenous $V\beta^{NOD}$ gene repertoire. Despite this marked perturbation in the T-cell–receptor gene repertoire, transgenic NOD mice developed autoimmunity similar to control littermates. Our data suggest that, in contrast to experimental models of autoimmunity in which correlations have been found between use of particular Vβ genes and autoimmune pathology (48), the T-cell–receptor β-chain gene repertoire does not alone determine susceptibility to autoimmunity in NOD mice.

FUTURE PROSPECTS

The transgenic mouse studies have been exceedingly useful for providing insights into fundamental questions of how immune tolerance is established and broken down, delineating the potential effector mechanisms involved in autoimmune destruction, and analyzing the underlying genetic defects contributing to disease pathogenesis. None of these transgenic mouse models, however, have faithfully reproduced the autoimmune process seen in NOD mice or humans. Important areas for future study include the following:

1. The development of efficient techniques to introduce genes directly into the germ line of NOD mice. In most published studies involving transgenic NOD mice, the transgene of interest has been transferred onto the NOD background by backcrossing. With this approach, genes adjacent to the transgene locus (that may protect against type I diabetes) are also initially transferred, and this combined with the mixed genetic backgrounds of the founders (typically F_1 or F_2 hybrid zygotes are used for microinjection) can complicate genetic analysis and necessitates repeated backcrossing and selection.

2. The development of techniques to target genes effectively, such as homologous recombination (49,50). With this technique, a vector carrying a selectable marker and nucleotide sequences identical to those of the DNA at the chromosomal site where the gene is to integrate is introduced into cells. The shared sequences help the vector find the desired location and exchange genetic material. In several laboratories, investigators have succeeded in using embryonic stem cells as a vehicle for gene transfer. These cells can be grown under selection and the targeted cells enriched; pure population of the relevant cells are then injected into mouse blastocysts, which are implanted into foster mothers. Chimeric animals are thus created in which both the host cells and the manipulated embryonic stem cells contribute to tissue formation.

With traditional transgenic techniques, it is not possible to control where transferred genes end up in the germ line. There is poor correlation between gene copy number and expression, and sometimes the transferred genes do not carry all the regulatory sequences needed for normal expression. Furthermore, analysis is usually limited to the gain of new functions. Homologous recombination, on the other hand, allows the introduction of new mutations and the endogenous wild-type genes to be disrupted. The ability to introduce specific mutations in situ allows the gene of interest to be left intact with its normal regulatory sequences and thus to be expressed in a manner identical to the endogenous gene.

3. The successful application of these technological advances to research on type I diabetes is contingent on continued new developments in the understanding of the disease pathogenesis. In particular, more precise localization of the genes conferring disease susceptibility and resistance and the identification and cloning of the authentic target autoantigens will enable the full potential of these powerful new techniques to be realized.

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