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CONSTRUCTION OF A RECOMBINANT PLASMID pSH-G CONTAINING THE RABIES-VIRUS GLYCOPROTEIN G GENE

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ABSTRACT: A plasmid named pSH-G was constructed with the rabies-virus G-gene insert. This plasmid was transfected into eukaryotic BHK-21 cells and its stability tested. The presence of the pSH-G plasmid was confirmed by means of polymerase chain reaction (PCR) after each of ten cell passages, and the results were positive. The stable BHK-21/pSH-G+ clone obtained can be used in the study of rabies as well as in the production of vaccines.

KEY WORDS: rabies, G gene, plasmids, transfection.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

The Rabies virus (RABV) is a member of the *Lyssavirus* genus of the Rhabdoviridae family, and is the etiologic agent of rabies, a disease that triggers fatal encephalitis. The RABV genome is composed of linear negative-sense RNA with 11932 nucleotides (nt) deduced from the fixed Pasteur-Virus sample (PV). It has five genes arranged in the order N (1350 nt), P (891 nt), M (606 nt), G (1572 nt) and L (6426 nt), and these transcribe the structural proteins with the same names (7). Like other RNA viruses, little replication fidelity is observed with the RABV because the L polymerase lacks exonuclease activity. This allows mutations to be incorporated, giving rise to genomic and antigenic variants with regional characteristics (2, 3).

The G gene has regions with a high diversity of nucleotides that are translated into G glycoproteins with structural diversity. This structural diversity is normally related to recognition of the virus receptors in the neurons as well as to stimulation of the immune system (1). Glycosylation of G glycoprotein is required for it to be immunogenic, and as this protein is responsible for stimulating the formation of neutralizing antibodies, advantage can be taken of its production in eukaryotic cells to make subunit vaccines (8). In addition, cDNA obtained from the G gene is potentially useful in the production of vaccines made from nucleic acids (12).

The aims of this study were to construct a plasmid containing the RABV G-gene insert (pSH-G), to transfect this plasmid into eukaryotic cells and to establish the stability of transfected clones by successive cell cultures. Materials and methods are described in Carnieli Jr. P. (4).The first stage in the construction of the pSH-G plasmid was the amplification of the G gene by reverse transcriptase (RT)-PCR for the production of the insert G. The fixed PV virus was used as a sample for amplification; this sample is used for vaccine production and is frequently used in rabies-diagnosis laboratories. The PV sample has genetic and antigenic characteristics similar to those of RABV isolates that circulate in most continents (6).

BHK-21 cells were chosen for pSH-G transfection because this cell lineage is extensively used in the study of RABV, including antigen production and serum neutralization of cell cultures (9). The extensive literature relating BHK-21 cells to RABV indicated that the presence of G gene was not expected to interfere in the cell metabolism, and this was confirmed during the course of this study.

The BHK-21 cells were transfected with pSH-G and then selected, as described elsewhere (4). Fifteen groups of viable cells (clones) were collected from the selection of transfected cells and expanded separately in a selective medium. During the selection period, the clones that proved to be viable were propagated, and after five weeks they were collected, registered by number from 1 to 15 and frozen in liquid nitrogen. First, however, a small sample of the 15 clones was separated to confirm the presence of the G gene by PCR using the same protocol as that used in previous steps. The PCR used to confirm the presence of transfected pSH-G in BHK-21 was designed to confirm the presence of the G gene and also exclude the possibility of the G1 and G2 primers amplifying sequences of cellular DNA. The β-actin gene was chosen as the reaction control because it is present in eukaryotic cells. As expected, the PCR result confirmed the presence of the G gene in the transfected cells.

The experiment to confirm the presence of the G gene and to exclude random amplification of the G primers was performed as follows: We used two different clones (cotransfected cells) registered as numbers 3 and 5, and nontransfected cells. First the RNA was extracted from the nontransfected cells and clones, and PCR was performed directly on the extracted RNA with the G1/G2 primers and the set of primers for β actin (here referred to as β1/β2). DNA was then extracted from the same clones and cells, and the extracted DNAs were subjected to PCR. Results of the amplifications with the β1/β2 primers were positive for both the extracted RNA and the extracted DNA. In parallel, PCR was performed with the same samples and the same protocol but using the G1/G2 primers.

The PCR with the G1/G2 primers was only positive in reactions using the DNA from clones because the G-gene insert bound to pSH is in the form of DNA. The results with the β1/β2 primers were positive in the clones and nontransfected cells both with RNA and DNA. In addition, the obvious difference in size between the amplified products of G gene (1572 nt) and those of β actin (289 nt) can readily be observed in the agarose gel. The PCR results described above confirmed that the G gene was transfected and that the G1/G2 primers do not amplify the cellular genome randomly (Figure 1).

To prove the integrity of the G insert, we sequenced the PCR product from clone 5. After alignment of the consensus sequence chromatogram generated with the PV-

sample sequence registered in GenBank under accession number M13215 on which the set of G1/G2 primers was based, the integrity of the cloned G insert was confirmed. The alignment showed that the insert shared practically 100% identity with the sequence registered in GenBank. With the exception of four synonymous point mutations, all the other nucleotides were identical. These four differences probably occurred because of the constant passage of the PV sample through mice brain to maintain the infective virus (1). They may also have occurred spontaneously. As the RABV is an RNA virus and therefore more susceptible to mutations compared with other DNA viruses (10), the presence of a number of synonymous point mutations, such as those described in this study, is acceptable. Based on the cloned sequence developed in this study, a nonradioactive probe for detecting the RABV G gene in clinical samples was produced and tested (5), confirming the integrity of the cloned G insert.

Transfection of the G gene in eukaryotic cells represents a significant achievement of this study, as transfection is essential for protein expression because G glycoprotein must have matured for it to be immunogenic. This is achieved by means of the metabolism of eukaryotic cells (11).

The aims of the present study were to construct a plasmid containing the RABV G-gene insert, to transfect this plasmid into BHK-21 cells and to test the stability of the clones produced. These objectives were achieved. The BHK clones transfected with pSH-G remained stable and viable throughout all the procedures described above and throughout all the cell cultures. The clones remained viable for the five months during which they were frozen, defrosted, propagated and frozen again in liquid nitrogen. The procedures described above were carried out every fifteen days on average, reaching a total of ten clone passages, during which the clones remained viable.

The main achievement of this study was the development and adaptation of techniques for constructing a plasmid with the RABV G insert and transfecting it into eukaryotic cells, thus opening up the possibility of cloning other genes of the RABV, a prospect which is of major biological interest. The results of this study confirm that a cDNA bank with sequences of interest protected from environmental selective pressures can be established using this methodology. The results also confirm that this methodology can be used in the study and production of DNA vaccines, allowing improved rabies control and prophylaxis in Brazil. The true achievement of this study,

however, was to confirm the stability of the pSH-G plasmid transfected into eukaryotic cells over time, proving that all the alternative proposals for the use of pSH-G are feasible.

It should be stated that all homemade products intended for biotechnological purposes are of great importance and interest to society, as they allow the operational costs of products for use in health care, livestock breeding and agriculture to be significantly reduced, thus generating social benefits. In summary, this study concludes that production of the pSH-G plasmid provides a further tool for use in the study of rabies.

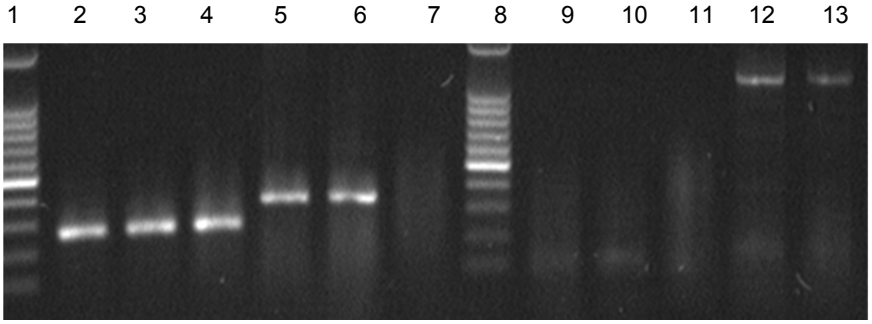

Figure 1. Lanes 1 and 8: 100bp molecular-weight marker; Lanes 2 to 7: amplifications of the β-actin gene using the β1/β2 set of primers; Lanes 9 to 13: amplifications of the G gene using the G1/G2 set of primers; Lanes 7 and 9: negative control (distilled water).

Samples used – Lane 2: RT-PCR of clone 3; Lane 3: RT-PCR of clone 5; Lane 4: RT-PCR of nontransfected BHK cells; Lane 5: PCR of clone 3; Lane 6: PCR of clone 5. Lanes 9 to 13: the samples are the same as in Lanes 2 to 7. Note Lanes 12 and 13 with amplifications of the transfected G gene and the difference in size between the RNA and DNA amplified products of the β-actin gene.

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