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Artículos sobre Técnicas

Conditions for Southern blot analysis for the detection of single copy genes: application to the screening for transgenic chickens

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SUMMARY

Southern blot analysis is one of the most widely used techniques in Molecular Biology. However, the detection of single copy genes requires the establishment of optimal conditions. In this paper we report on conditions for the detection of single copy genes by Southern blot analysis, and its application to the search for transgenic chickens in sperm-mediated DNA transfer experiments.

RESUMEN

El análisis por *Southern blot* es una de las técnicas más empleadas en Biología molecular. Sin embargo, para emplearla en la detección de genes de simple dotación genómica, es necesario optimizar algunas condiciones. En este trabajo nosotros reportamos condiciones que permiten la detección de genes de simple dotación genómica mediante la técnica de *Southern blot* y su empleo en el análisis para la búsqueda de pollos transgénicos en experimentos de transferencia génica mediada por espermatozoides.

INTRODUCTION

In experiments for the generation of transgenic animals it is absolutely necessary the use of Southern blot (Southern, 1975) for the analysis of newly incorporated DNA sequences. In most of the cases, the sensitivity of the method does not require the detection of single copy genes since multiple transgene sequences are usually found integrated into the host genome (Brem, 1989; Castro *et al.*, 1989; Limonta *et al.*, 1991; De la Fuente *et al.*, 1991).

Recently, sperm-mediated DNA transfer has been used for the generation of transgenic mice (Lavitrano *et al.*, 1989), and several groups have tried to reproduce their findings with so far negative results (Brinster *et al.*, 1989; De la Fuente *et al.*, 1990). However, some preliminary results obtained by us indicate that the method can probably still be used for the generation of transgenic farm animals (De la Fuente *et al.*, 1990; Pérez *et al.*, 1991). In these experiments we have noted that the transgene integration possibly occurs in very low copy number, those requiring the analysis for the detection of single copy genes (De la Fuente *et al.*, 1990).

In this paper we report on conditions for Southern blot analysis of single copy genes and its application to the screening for transgenic chickens in sperm-mediated DNA transfer experiments.

MATERIALS AND METHODS

Conditions for sperm-mediated DNA transfer in chickens

Forty eight hens (White Legorn) were inseminated employing 8 roosters from the same specie. The semen was collected from the roosters and mixed to prepare 400 μl fractions containing 50 μl semen, 350 μl PBS, and 2-5 μg DNA. These fractions were employed for hen insemination and each hen was inseminated twice a week (on Monday and Thursday) during 5 weeks. Before insemination, the fractions were incubated for 30 min at 30°C.

The DNA employed in the experiments consisted of a 6.9 kb fragment containing the 5' and 3' chicken ovoalbumin regulatory sequences, linked to the bovine prochymosin cDNA (Morales *et al.*, 1989; A. Pérez, unpublished results; Fig. 1).

DNA extraction

DNA was extracted from blood samples as described by Medrano *et al.* (1990).

Dot blot analysis

Ten μg DNA were denatured in 50 μl water by adding NaOH to a final concentration of 0.3 M, and incubating for 1 hour at 65°C. To the denatured DNA, 24 μl of 20xSSC were added, mixed and placed in ice. Then, the DNA mixture was blotted to a nitrocellulose membrane previously washing each well with 700 μl of 6xSSC. The membrane backing, prehybridization, and hybridization were carried out as described for Southern blot (see below).

Southern blot analysis

DNA (15 μg) were digested with 10 U/μg of BglII restriction enzyme (Enzibiot, Cuba) for 10 hours at 37°C in a reaction volume of 300 μl (100 mM NaCl, 10 mM Tris·HCl pH 7.4, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 100 μg/ml Bovine Serum Albumin). Digested DNA was treated with phenol:chloroform (1:1) and precipitated with ethanol for 10 hours at -20°C. Then, the precipitated DNA was rinsed with 1 ml of cool (-20°C) 70% ethanol, dried, redissolved in water, and

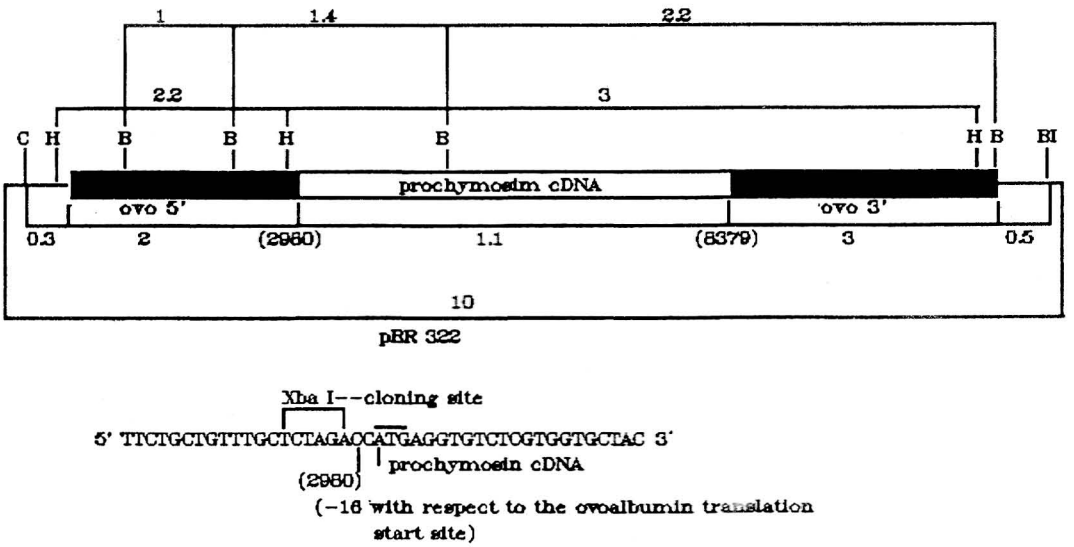

FIG. 1. Chimeric gene employed in the sperm-mediated DNA transfer experiments in chickens. The numbers indicated in parenthesis correspond to the chicken ovoalbumin (ovo) gene (Nucleotide sequences EMBL. ID: GGALB1, GGOVO3, GGOVAL; accesion numbers: JOO895, VOO382, VOO437, VOO438). The size of the fragments are in kb (upper line corresponds to the size of the BglII fragments; lower line corresponds to the size of the HindIII fragments). The sequence of the juncture between the ovoalbumin 5' sequences and the prochymosin cDNA is also indicated. Abbreviations: *C)* ClaI; *B)* BglII; *BI)* BglI; *H)* HindIII.

separated by electrophoresis in a 0.8% agarose gel run at 80 volts in TA buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.8).

The gel was soaked with gentle agitation in 250 mM HCl (2.5 ml/ml gel) for 15 min at room temperature, washed with deionized water, soaked again consecutively in denaturing (1 M NaCl, 0.5 M NaOH) and neutralizing (1.5 M NaCl, 0.5 M Tris·HCl, pH 7.4) solutions twice for 15 min each at room temperature, and then finally soaked in 20xSSC (3 M NaCl, 0.3 M Sodium Citrate, pH 7) for 15 min at room temperature. Nitrocellulose filters (BA85, Schleicher & Schuell) were prepared, and the DNA transfer done, exactly as described by Maniatis *et al.* (1982). Transfer was carried out for a period of 24 hours and the DNA was fixed to the filter by backing for 2 hours at 80°C in a vacuum oven (Yamato, Japan).

The filters were prehybridized during 2 hours at 42°C in a solution (100 μl/filter cm^2) containing 50% deionized formamide, 5xDenhardt's solution (1xDenhardt = 0.02% Polyvinylpyrolidone, Ficoll and Bovine Serum Albumin), 5xSSC, 50 mM $NaPO_4$ pH 7.4, and 150 μg/ml of heterologous (*E. coli*) DNA. Hybridizations were carried out in the same solution for 16 hours at 42°C, in the presence of 10^6 cpm/ml of a radiolabelled probe (specific activity > $5x10^8$ cpm/μg) prepared by labeling the prochymosin cDNA by the random priming technique (Feinberg and Vogetstein, 1984).

After hybridization, the filters were washed twice at 25°C in 1xSSC/0.1% SDS (1 ml/filter cm^2) for 10 min followed by single washes at 25°C in 0.1xSSC/0.1% SDS (1 ml/filter cm^2) for 15 min, and 68°C in 0.1xSSC/0.1% SDS (4 ml/filter cm^2) for 30 min. The filters were then subjected to autoradiography with intensifying screens at -70°C.

RESULTS AND DISCUSSION

From the total amount of laid eggs, 338 were incubated and 239 chickens were born. From them, 231 (114 hens and 117 roosters) were analyzed by Dot blot. In Dot blot analysis, in the first screening, around 36 samples gave signals with an intensity equal or higher than the single copy gene control (bovine DNA). These samples were further analyzed by Southern blot. As shown in figure 2,

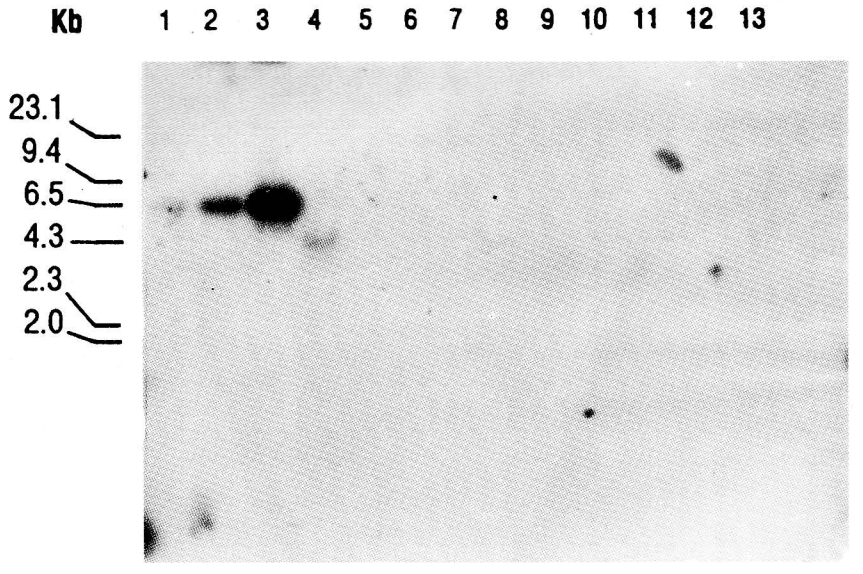

FIG. 2. Southern blot analysis of bovine and chicken DNA. The Southern blot analysis was carried out as described in *Materials and Methods* section. The probe used for hybridization was the radiolabeled bovine prochymosin cDNA (Morales *et al.*, 1989). *Lanes 1-3)* 0.02, 0.2 and 1 ng of the chimeric ovoalbumin/prochymosin gene, respectively; *lane 4)* 15 μg of bovine DNA digested with BglII; lanes 5-13) 15 μg of test chicken DNAs digested with BglII.

employing the protocol described in *Materials and Methods* section, it was possible to detect the prochymosin single copy gene in bovine DNA (figure 2, lane 4), and none of the analyzed animals were transgenic.

This permitted us to conclude that, with our experimental conditions and with the sensitivity obtained in Southern blot analysis, the sperm-mediated DNA transfer was not accomplished in chickens. However, as we have mentioned before (Castro *et al.*, 1990; De la Fuente *et al.*, 1990; Pérez *et al.*, 1991), it is important to study the sperm-mediated DNA transfer process before making any final conclusion about the possibilities of the method for DNA transfer in farm animals.

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