Colony hybridization method for screening in situ of eukaryotic amplified genes

Y. Malpièce, M.L. Michel and G. Carloni

Groupement de Génie Génétique, Institut Pasteur, 28 rue du Docteur Roux, F-75724 Paris Cedex 15 (France), and Centro Virus Respiratori, CNR, Policlinico Umberto I, via Regina Elena, Roma (Italy), 27 July 1982

Summary. A new method for autoradiographic screening of amplified genes in cellular clones is described. The main feature of the device is to keep viable cells from each clone, which can subsequently be regrown. The availability of this biochemical screening method allows screening for recombinants harboring unselectable markers as well.

At present, cloning prokaryotic or eukaryotic DNA sequences is generally carried out in *E. coli* using plasmid or phage vectors[1,2]. Eukaryotic vectors for cloning and propagating cellular genes in cell lines have only recently been described[3,4]. The use of recombinant vectors in eukaryotic cells is limited by the difficulty in screening for recombinants. This step is generally a daunting task when 100 (or more) colonies are to be cloned and examined. We report briefly a technique for rapid autoradiographic screening in eukaryotic colonies bearing transfected amplified hepatitis B virus 'S' gene sequences. This technique is based on autoradiography after colonies have been replica plated.

Material and methods. Cell lines and growth conditions. Mouse Ltk$^-$ aprt$^-$ cells, maintained in Dulbecco modified Eagle's medium with 10% calf serum, have previously been described[5]. The A29 cell line[6] (methotrexate-resistant chinese hamster ovary (CHO)) was maintained in growth medium supplemented with 3 times the usual concentration of non essential amino acids. This cell line contains at least 40 copies per genome of a mutated dihydrofolate reductase (DHFR) gene. Both these cell lines were kindly provided by R. Axel. A29 cells were grown in the presence of MTX (Sigma) at 40 µg/ml. L MTX^{40} HBs$^+$ clones (methotrexate resistant and Hepatitis B surface antigen producers) were obtained by cotransfecting Ltk$^-$ aprt$^-$ recipient cells with A29-DNA and with pAC-2 plasmid DNA. This plasmid containing HBV sequences has been described recently[7].

Cotransfection conditions were as described by Wigler et al.[6] and cotransfectants were selected in the presence of 0.2 µg/ml of MTX.

Transfected colonies resistant to 0.2 µg/ml of MTX and expressing HBs (to be published) were established for 9 passages (about three months) in this initial selection dose and then amplified by an initial plating in the presence of 2 µg/ml of MTX after cloning from Petri dishes.

Recombinant plasmids. The plasmid pDHFR 11 carrying a cloned cDNA copy of the mouse dihydrofolate reductase (DHFR) was obtained from Chang et al. (8). The plasmid pCP 10[9] and pAC carrying HBV sequences were obtained from P. Tiollais.

Replica plating of L clones MTX^{40} and A29 colonies to nitrocellulose filters. About 5,000–10,000 cells were plated in plastic Petri dishes (6 or 10 cm diameter) containing 6–15 ml of growth medium supplemented with MTX (10 or 40 µg/ml) and incubated at 37°C. After 15–20 days, 20–150 colonies per dish (of 1,000–5,000 cells each) were visible.

The medium was removed with a Pasteur pipette and cells washed once with trypsine (2.5 g/l) and EDTA (2 g/l) for 10–20 sec. At that time, most of the cells were refractive and rounded. After discarding the trypsin-EDTA, an overlay of 0.9% agarose (diluted with Dulbecco's modified Eagle's medium) was gently added to cover the bottom of the Petri dish. The solid overlay was carefully detached by means of a Pasteur pipette and transferred to the lid of the Petri dish. This transfer of colonies was monitored by looking through a microscope. In this manner, about 50–80% of the total number of colonies per plate could be easily transferred. We generally observed that the transfer of colonies is less efficient in the center of the Petri dish.

The original plates with residual cells of each colony were left at 37°C in the incubator to permit spreading of cells and subsequently refed with growth medium. Although the average of residual cells is quite variable from one colony to another, it is possible to obtain regrowth of the colonies. The transfer of colonies from the solid agarose surface to nitrocellulose filters (Schleicher and Schüll) was performed as previously described for bacterial colonies[10]: the filters were first laid on the agarose at 4°C *until* cells were adsorbed on them. Subsequent treatment was based on the method of Grunstein and Hogness[11] modified by Cami and Kourilsky[12]. Unless otherwise indicated, all subsequent steps were performed at 20°C in a small volume (2 ml). The cells were lysed for 5 min in 0.5 N NaOH and the denatured DNA was fixed by exposure for 10 min to 0.1 N NaOH–1.5 M NaCl. The filters were neutralized by washing with Tris-HCl (0.2 M pH 7.5)-EDTA (2 mM) for 2 min. Finally, LSSC buffer (2 × SSC-2 mM EDTA-25 mM NaH_2PO_4, pH 7.2) was added for 2 min. The filters were dried on 3 MM Whatman paper at room temperature for about 30 min and then overnight at 80°C.

Colony hybridization. Prehybridization was carried out for 6–12 h at 68°C in 6 × SSC-IX Denhardt's solution[13]. The filters were then transferred to 6 × SSC – 1 mM EDTA-IX Denhardt's solution with SDS at 0.5% for hybridization (3–5 ml per filter). About 5×10^5 cpm of ^{32}P nick-translated[14] pDHFR 11 and HBs gene DNA probes (sp. act. $\simeq 10^8$ cpm/µg) were used per filter. The HBs DNA was prepared by recovering from agarose gel the *Bgl*II restriction fragment of pCP10 plasmid. After 14–16 h of hybridization at 68°C, the filters were

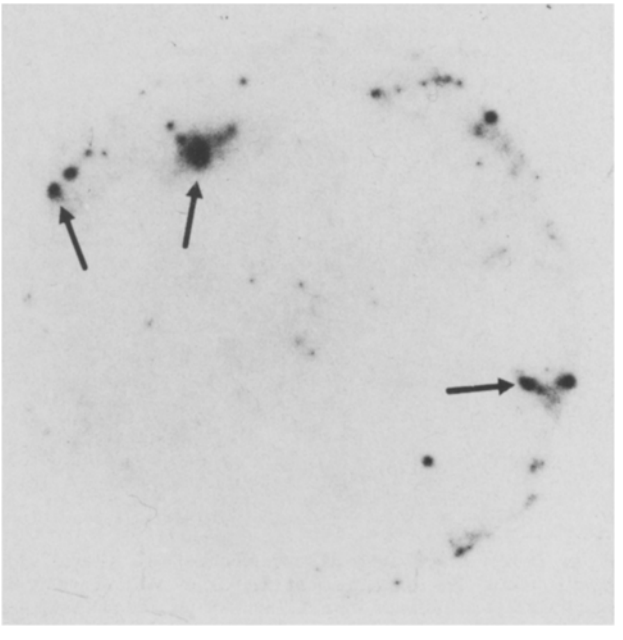

Figure 1. Autoradiogram of nitrocellulose filter obtained by agarose replica-plating from 60-mm dish (Falcon) seeded with L-HBs$^+$ MTX^{40} cell colonies, hybridized with ^{32}P nick-translated HBs-DNA (about 100 from 150 colonies were transferred). Arrows indicate typical spots.

washed 4 times in 200 ml 2 × SSC-IX Denhardt's solution − 0.5% SDS (30 min each at 68 °C) then twice 30 min at 20 °C in 2 × SSC. After blotting, the filters were dried for 1 h at 50 °C.

Cellular DNA was extracted and Southern blots were performed as previously described[15,16].

For dot blots, the high molecular weight DNA was resuspended in water and denatured for 10 min at 100 °C. The DNA was rapidly chilled in ice, diluted with 1 vol of 20 × SSC and spotted onto nitrocellulose preequilibrated with 10 × SSC with the minifold apparatus (Schleicher and Schüll). All subsequent steps were performed exactly as described above for replica plated colonies.

Exposure was for 1–3 days with either preflashed Royal X-Omat films with an intensifying screen at −70 °C or with No Screen films at 20 °C.

Results and discussion. As described by Wigler et al.[6], the drug methotrexate is a very potent tool for the selection of eukaryotic colonies bearing an amplified genetic marker. Following transfection of the DHFR gene, high doses of MTX allow selection of resistant clones harboring several copies of the gene coding for the enzyme dihydrofolate reductase which is normally inhibited by the drug. Recently, Southern blotting experiments have demonstrated that cotransfected sequences could be amplified along with the DHFR marker[6]. This amplification, however, appears to be variable and unpredictable. We describe here a method derived from the well-known Grunstein-Hogness technique[11] which allows a rapid screening of many clones.

L-mouse cell line was cotransfected with cellular DNA from A29 cell line (MTX®) and with the pAC-2 plasmid carrying HBs sequences. After several rounds of amplification with increasing doses of MTX (from 0.2 to 40 μg/ml), many clones resistant to 40 μg/ml of MTX were obtained (see 'Material and methods'). The transferred clones were then screened with a specific probe for HBs DNA sequences (fig. 1).

It is interesting to note the variation of intensity of specific signals. As the colony sizes are similar, this result strongly supports the idea that cotransfected sequences do not undergo the same extent of amplification when subjected to increasing MTX doses. The presence of amplified HBV sequences close to the DHFR marker in the genomic DNA of our colonies was also probed by Southern blotting (fig. 2). The cotransfected cells were amplified at 10 μg/ml of MTX. A typically amplified clone was screened by our method (lanes a and c). The cells were submitted to a new round of amplification at 40 μg/ml of MTX. A resulting subclone is presented in lanes b and d. Because the genomic DNA was cleaved with a restriction enzyme (*Hind*III), which does not cut within the HBV sequences[17], the appearance of several bands specific for HBV and DHFR suggests several integration sites (fig. 2). The clones were later found to produce HBs antigen by radioimmunoassay (results not shown). In addition, the specificity and dose effect of the HBs hybridization signals were evaluated by dot blot experiments (fig. 3). The DNAs of the amplified clones screened by our method give a strong signal in amounts as low as 250 ng, while a very weak signal, or none at all, is observed both with A29 DNA and with Ltk⁻ aprt⁻ DNA at amounts as high as 100 μg.

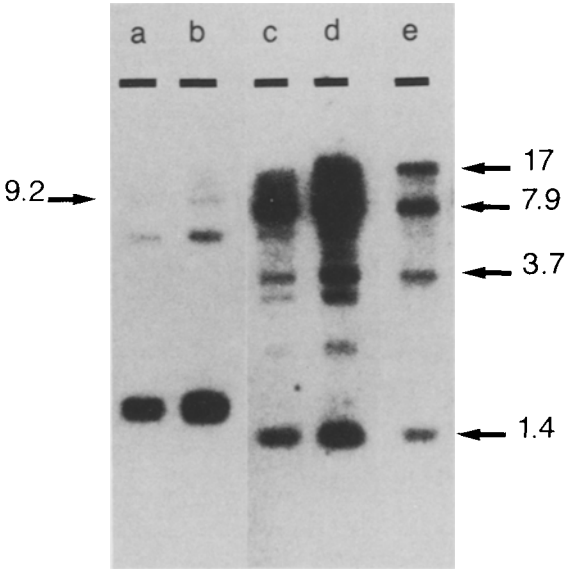

Figure 2. Blot hybridization of total cellular DNAs cleaved with *Hind*III from L-HBs$^+$ clones resistant to: 10 μg/ml MTX (a and c), 40 μg/ml MTX (b and d), and from A29 cell line (e). The DNAs (5 μg) were migrated through a 0.8% agarose gel and hybridized with ^{32}P nick-translated HBs-DNA (a and b). After dehybridization, the same blot was rehybridized with ^{32}P nick-translated pDHFR-11 DNA (c, d and e). The sizes of the DHFR marker in A29 DNA (lane e) are according to Wigler et al.[6], indicated in kilobases.

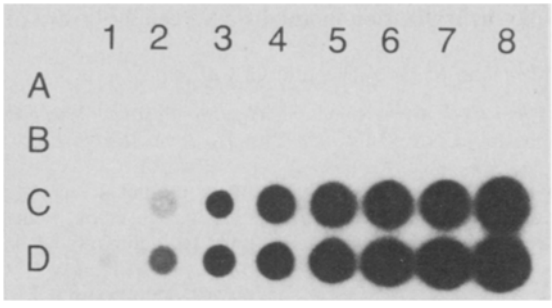

Figure 3. Dot blot hybridization of total cellular DNA with ^{32}P nick-translated HBs-DNA: *A* A29 DNA; *B* LTK⁻ aprt⁻ DNA; *C* L-HBs$^+$ resistant to 10 μg/ml MTX; *D* L-HBs$^+$ resistant to 40 μg/ml MTX. Amounts of DNA were as follows: *1* 25 ng; *2* 250 ng; *3* 1 μg; *4* 2,5 μg; *5* 5 μg; *6* 25 μg; *7* 50 μg; *8* 100 μg.

For positive clones the extent of amplification was in the range 100–200 copies per genome as demonstrated by Southern blotting (Michel et al., in preparation). These figures probably can be considered as a threshold for the detection of the relevant clones. Improved sensitivity might be obtained with the dextran sulfate hybridization method[18] or by the use of iodinated probes. Quite recently, Gerhard et al.[19] with iodinated probes were able to detect non-repeated sequences on chromosomes. On the other hand, our method appears to tolerate a considerable background.

We conclude that our method of in situ colony hybridization allows a rapid screening of an endogeneous or exogeneously introduced genetic marker in animal cells. At present, this is possible when the marker is present in many copies, i.e. amplified. The agarose replica plating used in our method allows one to keep parts of each colony, which can subsequently be regrown.

1 Benton, W.D., and Davis, R.W., Science *196* (1977) 180.
2 Mulligan, R.C., and Berg, P., Science *209* (1980) 1422.
3 Mulligan, R.C., and Berg, P., Molec. cell. Biol. *1* (1981) 449.
4 Berg, P., Science *213* (1981) 296.
5 Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G., and Chasin, L., Proc. natl Acad. Sci. USA *78* (1979) 3567.
6 Wigler, M., Perrucho, M., Kurtz, D., Dana, S., Pellicer, A., Axel, R., and Silverstein, S., Proc. natl Acad. Sci. USA *77* (1980) 3567.
7 Pourcel, C., Louise, A., Gervais, M., Chenciner, N., Dubois, M.F., and Tiollais, P., J. Virol. *42* (1982) 100.
8 Chang, A.C.Y., Numberg, G.H., Kaufman, R.J., Erlich, H.A., Schimke, R.T., and Cohen, S.N., Nature *279* (1978) 617.
9 Dubois, M.F., Pourcel, C., Rousset, S., Chany, C., and Tiollais, P., Proc. natl Acad. Sci. USA *77* (1980) 4549.

10 Hanahan, D., and Meselson, M., Gene *10* (1980) 63.
11 Grunstein, M., and Hogness, D.S., Proc. natl Acad. Sci. USA *72* (1975) 3961.
12 Cami, B., and Kourilsky, P., Nucleic Acids Res. *5* (1978) 2381.
13 Denhardt, D.T., Biochem. biophys. Res. Commun. *23* (1966) 641.
14 Rigby, P.J.W., Dieckmann, M., Rhodes, C., and Berg, P., J. molec. Biol. *113* (1977) 237.
15 Southern, E., J. molec. Biol. *98* (1975) 503.
16 Chenciner, N., Meneguzzi, G., Corallini, A., Grossi, M.P., Grassi, P., Barbanti-Brodano. G., and Milanesi, G., Proc. natl Acad. Sci. USA *77* (1980) 975.
17 Galibert, F., Mandart, E., Fitoussi, F., Tiollais, P., and Charnay, P., Nature *281* (1979) 646.
18 Wetmur, J.G., Biopolymers *14* (1975) 2517.
19 Gerhardt, D.S., Kawasaki, E.S., Bancroft, F.C., and Szabo, P., Proc. natl Acad. Sci. USA *78* (1981) 3755.

0014-4754/84/050483-03$1.50 + 0.20/0

Serum lectins from the scorpion *Vaejovis spinigerus* Wood bind sialic acids[1]

G.R. Vasta[2] and E. Cohen

Medical University of South Carolina, Department of Biochemistry, Charleston (South Carolina 29425, USA), and Roswell Park Memorial Institute, Department of Laboratory Medicine, Buffalo (New York 14263, USA), 27 June 1983

Summary. We have partially characterized the specificity of serum lectins from the scorpion *Vaejovis spinigerus* Wood. Agglutination, crossed-absorption and hemagglutination-inhibition patterns were similar but not identical to serum lectins from other members from the family Vaejovidae, and different from the Buthidae species studied so far. *V. spinigerus* serum lectins bind sialic acids and sialoconjugates, but also bind 2-keto-3-deoxyoctonate, uronic acids and N-acylaminosugars, all substances present in bacterial cell walls suggesting that they might be involved in defense functions.

Interest in the distribution and specificity of humoral lectins in the Subphylum Chelicerata (which comprises the classes Merostomata, Pycnogonida and Arachnida), a very conservative taxon in its evolutive aspects, motivated us to pursue a systematic study of the occurrence and serological properties of serum lectins from North American scorpions, whip scorpions and spiders (Arachnida). At present all arachnid species examined by us exhibited multiple serum lectins some of which are specific for sialic acids[3-7]. In this report we describe the serological characterization of serum lectins from the scorpion *Vaejovis spinigerus* Wood.

Material and methods. Scorpion sera: scorpions *Vaejovis spinigerus:* (18 males and 22 females, *V. confuscius:* 3 males and 2 females) were collected near Mesa, Arizona, and bled from pedipalps. Pooled scorpion hemolymph was allowed to clot at room temperature (25 ± 2 °C), the serum was cleared by centrifugation at 5000 × g for 15 min and stored at −25 °C.

Erythrocytes (RBC): Human blood samples were collected in ACD (citrate-dextrose). All other blood samples from diverse vertebrate species were obtained by venous or cardiac puncture and collected in Alsevers. RBC were washed twice with saline 0.85% and twice with tris-buffered saline: 100 mM tris-HCl, 50 mM NaCl, 10 mM $CaCl_2$, pH 7.6 (TBS), and suspended at concentrations of 5×10^6 RBC/ml in TBS.

Reagents: Neuraminidase from *Vibrio cholerae* (VCN) 500 U/ml and fetuin were purchased from Gibco, Grand Island, New York. N-acetylneuraminic acid β-methyl glycoside (NANA β MeGly) was kindly supplied by Dr W. Korytnyk, Roswell Park Memorial Institute, Buffalo, New York. All other reagents were purchased from Sigma Chemical Co., St. Louis, Missouri, at the highest purity available.

Enzyme treatment of RBC: RBC were treated with pronase P (P)(protease type VI from *Streptomyces griseus,* 3–4 units/mg) and VCN by the procedure of Uhlenbruck et al.[8]. P-treated RH_o(D) human RBC showed satisfactory agglutination titers with an incomplete anti-D serum (Ortho, Raritan, New Jersey). Human RBC were not agglutinated by *Limulus polyphemus* serum after VCN treatment.

Agglutination test: 5 µl of 2-fold serial dilutions of scorpion serum in TBS were placed in Terasaki 96 well trays (Robbins Scientific, Mountain View, California) and equal volumes of RBC suspension were added. The trays were vortex-mixed for 10 sec at speed 1 and incubated at room temperature for 45 min. Agglutination was read under the microscope and graded from 0 (negative) to 4+. Titers were recorded as the inverse ratio of the highest dilution showing a ½+ degree of agglutination. Controls for all titrations were the substitution of sera by TBS.

Desialylation of glycoproteins: glycoproteins were dissolved in 0.025 N H_2SO_4, 0.85% NaCl at 10 mg/ml. Hydrolysis was carried out for 1 h at 80 °C. Released sugars were analyzed by paper chromatography in ethyl acetate:pyridine:acetic acid: H_2O (5:5:1:3) for neutral sugars[9] using 1 µg of D-galactose, D-glucose, D-mannose (D-Man) and L-fucose (L-Fuc) as standards developing with the $AgNO_3$ and alcoholic KOH reagents[10], and in n-butanol:n-propanol: 0.1 N HCl (1:2:1) for sialic acids[11] using 1 µg of N-acetylneuraminic acid (NANA) and N-glycolylneuraminic acid (NGNA) as standards, developing with the resorcinol reagent. Sialic acids were the only sugars detected in the chromatograms. No neutral hexoses were released as measured by the anthrone reagent[12]. Released and total sialic acids were determined by the thiobarbituric acid[13] and resorcinol[14] methods respectively.

Percentages of released sialic acids were as follows: bovine submaxillary mucin (BSM): 72.1%; fetuin: 78.1%; PSM: 99.1%; thyroglobulin: 83.3%; bovine orosomucoid: 96.1%, and human orosomucoid: 98.3%. Hydrolyzed glycoproteins were exhaustively dialyzed against 0.85% NaCl and TBS, aliquoted and stored at −25 °C.

Hemagglutination-inhibition tests: Scorpion serum was diluted to 4–8 agglutination units/ml (1 agglutination unit is the amount of lectin present in the highest serum dilution which agglutinates with a degree of 2 in the scale from 0 to 4). All substances to be tested were dissolved in TBS (at concentrations up to 200 mM for mono- and oligosaccharides and 1% (w/v) for polysaccharides and glycoproteins) and brought to pH 7.6 with concentrated NaOH. Equal volumes of diluted sera and inhibitor solution were mixed and incubated for 45 min at room temperature. The mixtures were titrated as described before, with untreated or enzyme-treated RBC. Minimal concentrations required for the inhibition of two agglutination units were recorded. Controls were the substitution of