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Journal of Virological Methods 50 (1994) 245–255

Journal of Virological Methods

Recovery of a rare clone from a population of unstable retroviral vector-expressing mammalian cells using a new RNA extraction and slot-blot protocol

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Accepted 3 August 1994

Abstract

Although a useful and important method of gene transfer, retroviral vectors can be genetically unstable. In the course of experiments using DOEJS, a retroviral vector able to confer expression of a H-*ras* oncogene and a neomycin resistance gene (*neo*) on mammalian cells (Compere et al., 1989), it was found that the vast majority of infected rat embryo fibroblasts, recovered on the basis of *neo* activity (i.e., G418 resistance), did not express *ras* mRNA. It was subsequently observed that most cells in the $\psi 2$ cell line used to propagate DOEJS failed to produce virus capable of expressing both *ras* and *neo* in primary rat embryo fibroblasts. A simplified RNA extraction and slot-blot technique was developed to screen mRNA from several hundred fibroblast clones and, in doing so, infected fibroblast clones producing both *neo* and *ras* mRNA were identified at low frequency. The DOEJS/$\psi 2$ packaging line was subsequently subcloned and individual clones screened for their ability to confer appropriate gene expression on target cells. Subclone DOEJS/$\psi 2$-B6 was eventually isolated after screening 24 DOEJS subclones and 240 infected rat embryo fibroblast colonies. DOEJS/$\psi 2$-B6 was shown to induce reliably phenotypic transformation, G418 resistance, and *ras* and *neo* mRNA expression in primary rat embryo fibroblasts. The RNA extraction and screening procedure was thus useful for recovering an infrequent subclone producing a retrovirus with the original properties.

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SSDI 0166-0934(94)00123-5

Keywords: Retrovirus; Ras; RNA; Slot-blot; Genetic instability

1. Introduction

Cloning vectors employing replication deficient retroviruses are important tools for efficiently introducing genetic sequences into the DNA of mammalian cells. This technology has been used to insert foreign genes into embryos (e.g., Compere et al., 1989) and is increasingly being explored for human gene therapy (Fujiwara et al., 1993; Hasegawa et al., 1993; Oldfield et al., 1993). However, it is now being recognized that a limitation to the general usefulness of retrovirus-based vectors is their genetic instability (McLachlin et al., 1993; White and Bancroft, 1982). In this report, another example of such genetic instability is described and a technique that allowed us to recover clones of the original construct from a population composed primarily of viral variants is outlined. The DOEJS retroviral vector was originally obtained for the purpose of introducing an activated H-*ras* oncogene and a selectable drug resistance marker into primary rat embryo fibroblasts (REFs). This retroviral vector was constructed with a human c-H-*ras*-1 oncogene driven by a Moloney murine leukaemia virus long terminal repeat (Mo-MuLV LTR) and a Tn*10* neomycin resistance gene (*neo*) driven by a SV40 early-region promoter (Compere et al., 1989) conferring resistance to the drug G418 sulfate. The replication defective retrovirus was maintained in a $\psi 2$ packaging cell line. When virus was harvested from the $\psi 2$ culture supernatant and used to infect REFs, it was found that essentially none of the G418-resistant REFs was morphologically transformed, although most should have been if the *ras* oncogene were active. A rapid RNA extraction and slot-blot procedure was designed to recover rare clones in the infected REF population that expressed both *neo* and *ras* and to isolate a functional DOEJS/$\psi 2$ clone capable of producing appropriate virus.

2. Materials and methods

2.1. Cells, retrovirus and culture conditions

Third passage REFs were prepared as described earlier (Bennett et al., 1993). DOEJS/$\psi 2$, kindly provided by Dr. R. Jaenisch, is a $\psi 2$ cell packaging line that produces a replication deficient retrovirus containing the DOEJS construct (Compere et al., 1989). The DOEJS construct includes the EJ bladder carcinoma human c-H-*ras*-1 oncogene driven by a Mo-MuLV LTR and a Tn*10* neomycin resistance gene driven by a SV40 early-region promoter. DOEJS/$\psi 2$ cells and REFs were cultured in Dulbecco's minimal essential medium (DMEM) plus 10% fetal calf serum (FCS) (Gibco/BRL) at 37°C in a 5% CO_2/95% air atmosphere.

2.2. Retrovirus-mediated gene transfer

Virus particles were harvested from the supernatant of 90% confluent DOEJS/$\psi 2$ cells, 24 h after medium change. The supernatant fluid was filtered through a 0.45 μm

filter to remove cell debris. REFs, seeded at 5×10^5 cells per 10 cm dia. tissue culture dish 24 h prior to infection with retrovirus, were exposed for 18 h to virus in 5 ml of DOEJS/ψ2 supernatant containing 8 μg/ml polybrene. The medium was then diluted with fresh DMEM + 10% FCS to reduce the polybrene concentration to 1.6 μg/ml and incubation was continued for an additional 48 h. Cells were detached by trypsinization and cells were replated at 1×10^5 per 10 cm dia. tissue culture dish. Infected REFs were grown for 10 days in complete medium containing 400 μg/ml G418 sulfate (GIBCO/BRL) with twice weekly feedings. For Northern analysis, G418-resistant colonies were cloned from the population of DOEJS-infected REFs and grown in selection medium until a suitable cell density was obtained. For slot-blot analysis, DOEJS-infected REF clones were plated in 24-well tissue culture dishes in selection medium until the time that nucleic acids were extracted.

2.3. Extraction of total cellular RNA

For Northern blot analysis (*standard* protocol), RNA was extracted from 5×10^6 cells as described previously (Birnboim, 1988; Birnboim, 1993).

For slot-blot analysis (*slot-blot* protocol), the extraction procedure was simplified to eliminate precipitation and DNA removal steps. Cultures containing 4×10^3 to 5×10^5 REFs per well in a 24-well culture plate were washed with a balanced salt solution before addition of 250 μl RNA extraction solution (RES) containing 25–75 μg/ml proteinase K (depending on the cell number). RES is 0.5 M LiCl, 1 M urea, 0.25% SDS, 20 mM sodium citrate, 5 mM CDTA, pH 6.8, modified slightly from (Birnboim, 1988). Plates were maintained at 50°C with shaking until the digested material was clear and could be easily pipetted ($\leq$ 2 h). Extracts were then transferred to 2 ml microcentrifuge tubes and individually sonicated with a 1/8″ microprobe at low power. Samples were extracted sequentially with 50 μl phenol/chloroform and 100 μl chloroform, leaving behind the organic (bottom) phase and interface material. The final volume of the aqueous samples was approximately 200 μl. Contaminating DNA was not removed for reasons described below. Samples were analyzed in duplicate (100 μl), except where very small amounts of material were available. Traces of chloroform were removed by evaporation to avoid damaging the plastic slot-blot apparatus.

2.4. Northern and slot-blot analyses

Northern analysis (of RNA prepared by the *standard* protocol) was carried out essentially as described (Birnboim, 1988). Briefly, purified total RNA (approximately 10 μg) was denatured with formaldehyde, separated electrophoretically on a 1.2% agarose gel containing 0.2 M formaldehyde, and transferred to a Hybond-N nylon membrane filter (Amersham) using a vacuum system. For slot-blot analysis (of RNA prepared by the *slot-blot* protocol), 100 μl samples, prepared as described above, were mixed with 10 μl 37% formaldehyde and heated for 30 min at 65°C. Contaminating double-stranded DNA is not expected to be denatured under these conditions and hence not expected to bind in significant amount to membrane filters. Hybond-N membranes were hydrated for 15 min in 0.5 M–2.5 M of either NaCl, LiCl, CsCl, or KCl, or in 0.5–10 $\times$ SSC

(10 × SSC: 1.5 M NaCl, 0.15 M sodium citrate) and mounted in a BioRad slot-blot manifold. 100 μl of the corresponding high salt solution was added to the denatured RNA samples immediately prior to transfer. A 150 W heat lamp was used to maintain an elevated temperature in the manifold throughout the transfer to avoid precipitation of detergent. Samples were applied under 40 cm water of vacuum and wells were washed once with 200 μl 10 × SSC. Membranes were removed from the slot-blot apparatus, air-dried, heated for 20 min at 80°C, and irradiated with ultraviolet light for 3 min. Pre-hybridization and hybridization was carried out as described (Chen and Birnboim, 1993).

The DNA probes used were a 2.4 kb *Bam*HI/*Hin*dIII fragment of the pSV2 plasmid containing the neomycin resistance gene (*neo* probe) (Southern and Berg, 1982), a 2.9 kb Sac1 fragment from the pEJ6.6 plasmid containing the human c-H-*ras* gene (*ras* probe) (Shih and Weinberg, 1982) and a 1.2 kb *Pst*I fragment from the pGAPDH plasmid containing the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (GAPDH probe) (Piechaczyk et al., 1984). Probes were labelled with [^{32}P]dCTP using a random primer labelling kit (Pharmacia). At the end of the hybridization procedure, membranes were air-dried and exposed to X-ray film (Kodak) for 3–48 h at −100°C. When membranes were re-probed, the first probe was removed by heating the membranes in 0.25% SDS, 10 mM sodium citrate, pH 6.8, at 95°C for 5 min.

3. Results

3.1. Conditions to maximize binding of RNA to the slot-blot membrane

Effective binding of RNA to membranes requires high salt and is improved by denaturation (Thomas, 1983). The concentration of LiCl salt present in RES was not high enough to permit optimal binding of RNA; however, raising the salt concentration above 0.5 M caused precipitation of dodecyl sulfate. Substitution of sarkosyl, a more soluble detergent, for SDS was not as effective in extracting RNA (data not shown). Several monovalent cations were tested for their ability to promote binding of RNA to nylon membrane, including 0.5–10 × SSC or 0.5–2.5 M (final concentration) NaCl, LiCl, CsCl, and KCl. LiCl, NaCl, or CsCl added to RES were ineffective in promoting RNA binding. Only KCl (at 1.5–2.5 M final concentration) was effective in promoting RNA binding. Although the potassium salt of dodecyl sulfate is known to be very insoluble at low temperature (Birnboim, 1983), we found that it was soluble at elevated temperature. Precipitation was avoided if samples were maintained at 55–65°C in a water bath both prior and subsequent to the addition of KCl (final concentration, 2.5 M) and the slot-blot apparatus was heated with a 150 W lamp.

The optimal conditions (*slot-blot* protocol) for binding of RNA to nylon membranes are as follows: preheated, formaldehyde-denatured sample in RES are mixed with an equal volume of preheated 5 M KCl and maintained at 65°C. Membranes are hydrated in 10 × SSC. Diluted samples are quickly transferred to the heated slot-blot apparatus. After the sample is applied to the membrane, the well is washed with 200 μl 10 × SSC.

3.2. Capacity of the nylon membrane

Total RNA was extracted from REFs using either the *standard* or *slot-blot* protocol. Highly purified RNA prepared by the *standard* protocol was dissolved either in CCS (1 mM sodium citrate, 1 mM CDTA, 0.1% SDS, pH 6.8) or in RES plus indicated salts and applied to a nylon membrane using the slot-blot apparatus. RNA prepared using the *slot-blot* protocol from different numbers of REFs (4×10^3 to 5×10^5) was also transferred to a membrane. A ^{32}P-labelled GAPDH probe was used to monitor mRNA binding which was measured by densitometric analysis of an autoradiograms. Densitometric analysis revealed a linear dose response relationship between the amount of RNA loaded and the intensity of the GAPDH signal (Fig. 1). The lower limit for detection of GAPDH mRNA in this experiment was 200 ng of total RNA per slot. The upper limit of linearity was 3 μg of RNA per slot. The maximum capacity of the membrane for RNA was also assessed by using a double thickness of membrane, and staining the bottom membrane with 0.1% methylene blue in 50% ethanol. Overloading of the first membrane (i.e., recovery of RNA to the second membrane) was observed at amounts greater than 3 μg per slot (9 mm^2 surface area of membrane). The signal from 200 ng to 3 μg RNA corresponded to the signal obtained from approximately 8×10^3 to 1.2×10^5 cells extracted using the *slot-blot* protocol. In terms of binding of pure RNA to nylon membrane, equivalent results were obtained using either CCS or RES as the RNA solvent prior to addition of high salt.

3.3. Screening of retrovirally infected REFs for mRNA production

Five REF populations were infected using supernatant from the DOEJS/ψ2 producer line as described. Morphological transformation, characteristic of H-*ras* expressing cells, could not be detected in any of the G418-resistant populations (Fig. 2). By Northern analysis, all of the G418-selected populations could be shown to express *neo* mRNA in the absence of detectable levels of *ras* mRNA (Fig. 3). It thus appeared that the *ras* gene was either inactivated or lost. In order to determine whether any cells in the selected populations expressed *ras*, 130 individual G418-resistant clones were isolated and analyzed using the *slot-blot* protocol. Only 1 clone (REF-E2) was found to express both the *neo* gene and an activated *ras* oncogene; it was also morphologically transformed (Fig. 2).

In an attempt to re-establish a stable DOEJS/ψ2 producer line capable of reliably infecting REFs, 24 DOEJS/ψ2 clones were isolated and grown to a suitable density for further study. The culture supernatant from these clones was used to infect REFs. Ten G418-resistant REF clones were isolated from each infection (a total of 240 clones). Of the 24 DOEJS/ψ2 clones examined, only 1 (DOEJS/ψ2-B6) was found to reliably introduce both a functional neomycin resistance gene and a *ras* oncogene into cells (Fig. 4). All of the 10 G418-resistant REF clones isolated following infection with DOEJS/ψ2-B6 supernatant expressed *ras* mRNA and were morphologically transformed (Fig. 2).

Given the potential instability of retrovirally transfected genes, minimizing the cell number required for mRNA analysis may minimize the time for variability to develop.

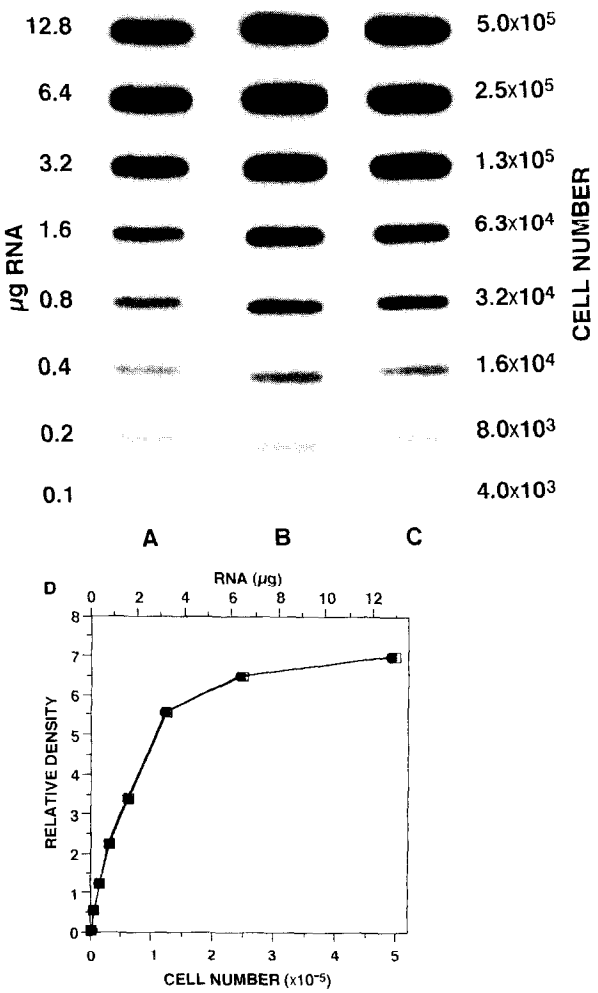

Fig. 1. Comparison of total cellular RNA extracted using the *standard* or the *slot-blot* protocols. Panel A, *standard* extraction dissolved in CCS; Panel B, *standard* extraction dissolved in RES; Panel C, *slot-blot* extraction dissolved in RES. The RNA samples were applied to a nylon membrane and probed with a 1.2 kb *Pst*I fragment of pGAPDH (GAPDH probe) as described in Materials and methods. Panel D, densitometric analyses of panels A-C: × *standard* extraction, dissolved in CCS;, □ *standard* extraction, dissolved in RES; ●, *slot-blot* extraction, dissolved in CCS.

To establish the minimum time needed to (1) introduce a gene into a cell, (2) clone individual transformants, and (3) analyze mRNA with the *slot-blot* protocol, REFs were infected with DOEJS/ψ2-B6 cell supernatant. Morphologically transformed G418-resistant colonies were isolated with cloning rings ten days after infection and plated in two wells of a 24-well plate. One was used to maintain viable stocks and the other was grown for up to 15 days for RNA extraction (approximately 50% confluency or about 5×10^4 cells/well). This provides approximately 1.3 μg RNA (sufficient for 2

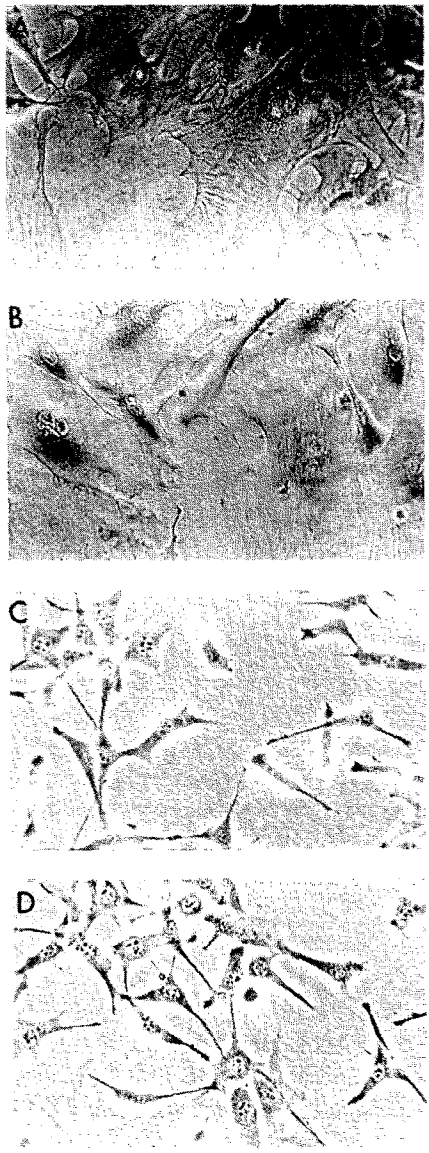

Fig. 2. Photomicrographs of untreated and DOEJS/ψ2 infected REFs. Panel A, untreated REFs; panel B, a representative DOEJS-infected REF clone expressing the neomycin (G418) resistance gene in the absence of detectable H-*ras* mRNA; panel C, the only DOEJS-infected REF clone (REF-E2), identified amongst 130 clones isolated, that expressed both *neo* and activated H-*ras* mRNA (see Figs. 3 and 4); panel D, a representative DOEJS/ψ2-B6-infected REF clone expressing both *neo* and activated H-*ras* mRNA. Magnification 280×.

analyses). Using the *slot-blot* protocol, confirmation of the presence of *ras* mRNA in selected clones was achieved in about 25 days from the time of initial gene transfer.

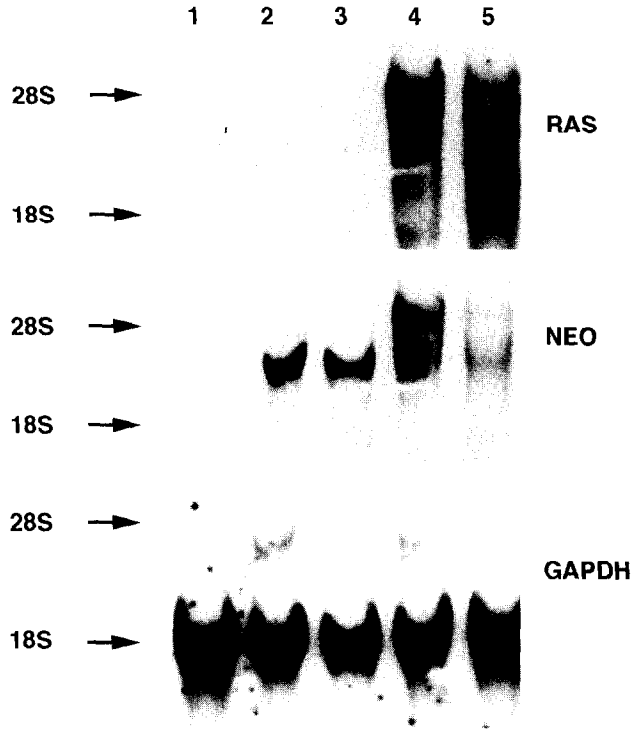

Fig. 3. Northern blot of RNA isolated from control and DOEJS/ψ2-infected REF populations. RNA was extracted using the *standard* procedure and probed with a *ras* probe, a *neo* probe, or a GAPDH probe as described in Materials and methods. Lane 1, REFs; lane 2, DOEJS/ψ2-infected REF population #1; lane 3, DOEJS/ψ2-infected REF population #2; lane 4, DOEJS/ψ2-infected clone REF-E2; lane 5, DOEJS/ψ2 cells (producer line).

4. Discussion

Genetic instability associated with the use of retroviral vectors is now well recognized (McLachlin et al., 1993; White and Bancroft, 1982). Instability was encountered when an attempt was made to use DOEJS retrovirus (Compere et al., 1989) to introduce an activated H-*ras* oncogene into REFs; instability was manifest initially as a failure to cause phenotypic transformation of cells resistant to G418. The lack of H-*ras* mRNA expression was confirmed by Northern analysis of the transfected populations. In order to isolate a clone that produced virus with the original *neo/ras* genotype from the apparently unstable population of DOEJS/ψ2 cells, an RNA extraction/slot-blot protocol was developed to allow rapid screening of several hundred infected REF clones. One DOEJS/ψ2 clone from the original population (DOEJS/ψ2-B6) was identified with the ability to propagate virus that could reliably cause morphological transformation, G418 resistance, and production of *ras* and *neo* mRNA in REFs.

The procedure allows use of a relatively small number of cells as a source of RNA by minimizing the number of steps involved in the RNA purification. Ethanol precipitation

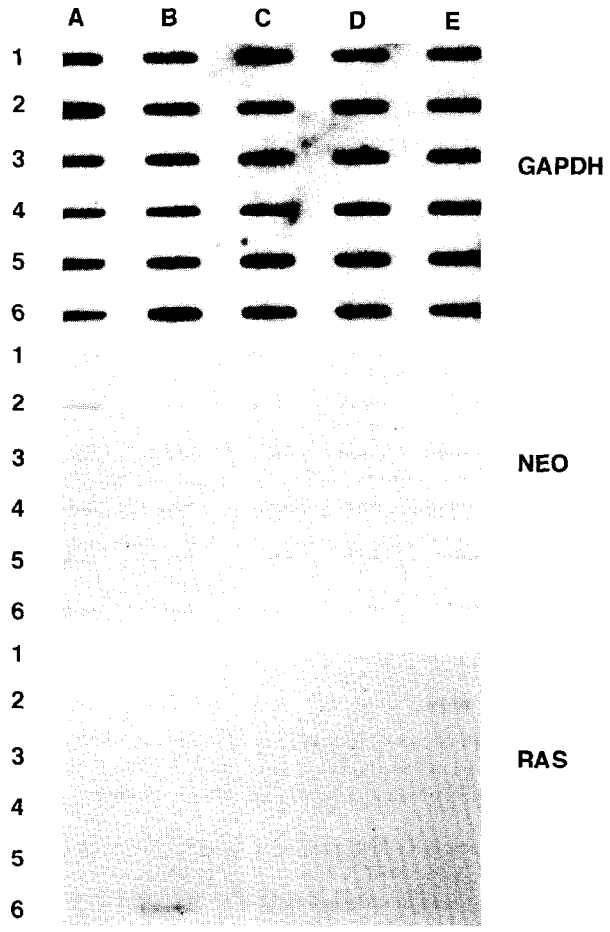

Fig. 4. Selection of a DOEJS/ψ2 clone capable of conferring *ras* expression on REF cells. RNA was extracted using the *slot-blot* protocol from G418-resistant REF clones which had been infected with virus from 24 different DOEJS/ψ2 clones. Each slot (A1 to D6) represents RNA from 1 of 10 REF clones infected with virus from each DOEJS/ψ2 clone. This slot-blot is representative of 10 such analyses, in which 10 different REF clones were screened. In the case of DOEJS/ψ2 clone B6, 10 of 10 REF clones expressed *ras* mRNA; none of the other DOEJS/ψ2 clones produced virus capable of conferring *ras* expression in any REF clone tested. Virus from the parental DOEJS/ψ2 population was only rarely able to confer *ras* expression on REF cells (1 out of 130 REF clones screened). Slots E1–E6 represents RNA from 6 of these 130 clones, including REF clone E2 which was positive for *ras*. The same membrane was hybridized with a *ras* probe, a *neo* probe, and a GAPDH probe, as described in Materials and methods.

is avoided and no attempts are made to remove DNA since double-stranded DNA is not expected to bind to the nylon membrane under the conditions employed. A high concentration of K^+ was found to be most effective in promoting binding of RNA in RES to the nylon membrane. The expected precipitation of the potassium salt of dodecyl sulfate was avoided by maintaining solutions at 50–60°C. A number of other slot-blot

techniques have been developed to allow extraction of a RNA from a small number of cells and direct application to membranes (Bresser et al., 1983; Cheley and Anderson, 1984; Manser and Gefter, 1984; Varela-Echavarria et al., 1993). However, each has limitations such as variability in the efficiency of trapping of mRNA (Thompson and Gillespie, 1987) and requirement for isolation of cytoplasm. Our protocol permits rapid, efficient isolation of RNA and direct application to membrane without intermediate precipitation steps. The RNA extraction and screening procedure proved useful for recovering an infrequent DOEJS/ψ2 subclone (DOEJS/ψ2-B6) producing a retrovirus with original properties.

Acknowledgements

The expert technical assistance of Cai-Ying Zou and Chantal Couture is gratefully acknowledged. S.A.L.B. is a recipient of a Medical Research Council of Canada studentship. This work was supported by a grant from the Medical Research Council of Canada to H.C.B. H.C.B. is a Career Scientist of the Ontario Cancer Treatment and Research Foundation.

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