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Integrated simian virus 40 sequences in transformed cell DNA: Analysis using restriction endonucleases

(agarose gel electrophoresis/transfer of DNA to membrane filter/DNA·RNA hybridization)

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ABSTRACT **The DNAs from five independent simian virus 40 (SV40) transformants of the BALB/c 3T3 mouse cell line were digested with either the *Hpa*II or the *Bam*HI restriction endonuclease and the resulting fragments were fractionated by gel electrophoresis. The DNA fragments were denatured *in situ* in the gel and transferred to a membrane filter. Fragments containing viral DNA were detected by hybridization with high specific activity ^{32}P-labeled SV40 complementary RNA (cRNA) synthesized *in vitro*. Each of the lines yielded a small number of fragments containing SV40 DNA and the fragments from each line were different. This observation shows that the structure of the integrated SV40 DNA and/or its location in the host DNA are different in each line.**

A fundamental feature of virus-transformed cells is the persistence of viral genetic information (1–12). In the cases which have been carefully studied to date, the persistent viral genes appear to be covalently linked to host genes (i.e., "integrated"), and expression of one or more integrated viral genes seems to be required for the maintenance of the transformed phenotype (13–27). Little is known at present about the detailed structure of integrated viral DNA or about the molecular mechanisms involved in integration. This is largely due to the fact that viral DNA represents a very small fraction (about 10^{-6}) of transformed cell DNA.

In the past several years a good deal of information has accumulated about the structure and function of the genome of the papovavirus simian virus 40 (SV40). Much of this information has been acquired using site-specific bacterial restriction enzymes to dissect the viral DNA and construct detailed physical maps (28). In this paper we show that similar techniques can be used to study the structure of integrated SV40 DNA. Our approach is analogous to that previously employed by Botchan and McKenna (29) in a study of the SV40-transformed line SVT2. DNA from SV40-transformed cells was cleaved with a restriction enzyme and the resulting fragments were fractionated by agarose gel electrophoresis. The fragments were then denatured *in situ* in the gel, transferred directly to a membrane filter, and hybridized with high specific activity SV40 [^{32}P]cRNA synthesized *in vitro* (6, 40, 41). This method made it possible to locate the restriction fragments containing SV40 DNA and estimate their molecular weights. The SV40-transformed lines which were studied represented several independent clones obtained following infection of BALB/c 3T3 cells with SV40 at low multiplicities of infection. After digestion of transformed cell DNA with either the *Hpa*II or *Bam*HI restriction endonuclease, it was found that the pattern of fragments containing viral DNA was different for each transformed line. This finding indicates that the structure of the integrated SV40 DNA and/or its location in the host genome is also different in each transformed line.

Abbreviations: SV40, simian virus 40; cRNA, complementary RNA; SSC, standard saline citrate; PBS, phosphate-buffered saline; MEM, Eagle's minimum essential medium.

MATERIALS AND METHODS

Virus and Viral DNAs. SV40 virions (small plaque, strain 776) were purified and the viral DNA was extracted as previously described (30). SV40 DNA labeled with [^{32}P]orthophosphate was prepared by the method of Hirt (31) as modified by Danna and Nathans (32).

Cells and Cell DNAs. BALB/c 3T3 (A31^{7}) cells were obtained from G. Todaro and propagated on Eagle's minimum essential medium (MEM) with 20% fetal calf serum. A number of independent SV40-transformed cell lines were derived from these cells by a method similar to that of Todaro and Green (33). BALB/c 3T3 cells growing in 1.6 cm microwells (Linbro) were infected with SV40 (small plaque, strain 776) at a multiplicity of 0.5 PFU per cell after the cells had reached approximately 90% confluence. Twelve to 24 hr after infection the cells were trypsinized and the contents of each microwell were plated in a 100 mm petri dish. Thereafter, the cells were maintained on Eagle's minimum essential medium supplemented with 10% fetal calf serum (MEM-10) which was changed at weekly intervals. Colonies of transformed cells recognizable by their density and altered morphology were first visible at about 2 weeks after infection. The average number of transformed colonies per dish was about three. No transformed colonies were observed in five control dishes containing mock-infected cells. At 3–4 weeks after infection transformed colonies were surrounded by glass cloning cylinders, trypsinized, and plated in 1.6 cm microwells containing MEM-10. To insure the independence of the transformed lines, only one colony was harvested from each petri plate. After the transformed cells had grown to confluence in the microwells, they were trypsinized, replated at high dilutions, and allowed to grow into macroscopically visible colonies. A single well-isolated colony of each transformed line was selected and harvested with a cloning cylinder as described above. This cloning procedure was then carried out two more times. All of the transformed lines obtained were found to express SV40 T antigen by the indirect fluorescent antibody method and to contain SV40 DNA detectable by DNA·RNA hybridization. Each line was given the designation SVB followed by a strain number, e.g., SVB201.

DNA was isolated from BALB/c 3T3 cells and various SV40-transformed lines by a modification of the method of Thomas *et al.* (34). Cells were grown to confluency on MEM-10 in 32 ounce (0.95 liter) prescription bottles. After removal of the medium, the cells were rinsed twice with phosphate-buffered saline (PBS)-EDTA (8 g/liter of NaCl,

0.2 g/liter of KCl, 0.9 g/liter of $Na_2HPO_4 \cdot 2H_2O$, 0.2 g/liter of KH_2PO_4, 0.19 g/liter of disodium ethylenediaminetetraacetate) and then incubated at room temperature with 10 ml of PBS-EDTA until they detached from the surface of the bottle. The cells were collected by centrifugation and resuspended in PBS-EDTA. Pronase (final concentration 1 mg/ml) and sodium dodecyl sulfate (final concentration 0.5%) were added to the cell suspension and the resulting lysate was incubated overnight at 37° with gentle shaking. The lysate was extracted twice with an equal volume of phenol saturated with 0.1 M Tris·HCl pH 8.6, and the residual phenol was removed from the aqueous phase by repeated ether extractions. Pancreatic RNase (Calbiochem) previously heated to 80° for 10 min to destroy DNase was added to a final concentration of 20 μg/ml and the resulting solution was incubated at 37° for 2 hr. Following removal of the RNase by phenol extraction (three times), the DNA was collected by ethanol precipitation, redissolved in a small volume of 0.01 M Tris·HCl; 0.001 M EDTA, pH 8.6, and dialyzed exhaustively against the same buffer.

Cleavage of Cell DNA with Bacterial Restriction Enzymes. Endonuclease R·*Hpa*II (35, 36) was a gift of Dr. Michael Mann and endonuclease R·*Bam*HI (37) was purchased from Bethesda Research Laboratories, Inc. In both cases the DNA concentration in the reaction mixtures was 500 μg/ml or less. The amount of enzyme required for complete digestion was determined in trial reaction mixtures which contained marker SV40 DNA in addition to the cell DNA to be digested, and in all experiments at least twice that amount of enzyme was used. Digestions with the *Hpa*II enzyme were carried out at 37° for 6 hr in 70 mM Tris·HCl, pH 7.5, and 7 mM $MgCl_2$. Digestions with the *Bam*HI enzyme were carried out at 37° for 6 hr in 6 mM Tris·HCl, pH 7.4, 6 mM $MgCl_2$ and 6 mM 2-mercaptoethanol.

Agarose Gel Electrophoresis and Transfer of DNA to a Membrane Filter. Electrophoresis of DNA was carried out in 1% agarose (SeaKem) vertical slab gels in a buffer containing 40 mM Tris·HCl, pH 7.8, 5 mM sodium acetate, and 1 mM EDTA at 1 V/cm for 15 hr. A gel chamber similar to those described by DeWachter and Fiers (38) and Reid and Bieleski (39) was used. The gel slabs measured 12 × 13 × 0.9 cm and were supported with a 3 cm high plug of 12% polyacrylamide. In experiments carried out to determine the capacity of the gels for DNA, various quantities of BALB/c 3T3 DNA fragments (prepared by digestion with endonuclease R·*Hpa*II) were mixed with a small quantity of ^{32}P-labeled SV40 form II (nicked circular) DNA and electrophoresed as described above. It was found that 20 μg of cell DNA fragments could be accommodated in a slot 0.6 cm wide (0.37 $\mu g/mm^2$) without significantly affecting the mobility or band-width of the SV40 marker.

After electrophoresis the DNA was denatured and transferred to a nitrocellulose filter by a method similar to that of Southern (41). [A technique similar in principle but different in detail has also been developed by Shinnick *et al.* (50).] The gel was immersed in a solution of 1 M KOH for 20 min at room temperature to denature the DNA. The KOH was then neutralized by addition of an equal volume of 1 M Tris·HCl, pH 7, 1 M HCl and the incubation at room temperature was continued for 60 min. The gels were then immersed in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 6.8) for 10–20 min.

The denatured DNA was transferred from the gel to the membrane filter by placing the gel on a slightly larger sheet of Millipore type HAWP00010 membrane filter material (prewet with 6 × SSC) on top of about five layers of Whatman No. 1 paper. The gel was covered with Saran Wrap and allowed to dry down to a thin layer about ⅕ to ⅒ of its original thickness. This generally required about 4 hr. To facilitate the drying process the Whatman No. 1 sheets were changed every 15–30 min and during the last 2 hr the gels were weighted with a sheet of plate glass. During this period fluid flowed out of the gel, through the membrane filter and into the Whatman No. 1 sheets. Denatured DNA dissolved in this fluid bound quantitatively to the membrane filter material (40). After drying was complete the membrane filter sheet was peeled from the gel, rinsed briefly in 6 × SSC, and dried *in vacuo* for 2 hr at 80° (40). In a control experiment the efficiency of transfer of ^{32}P-labeled SV40 form II DNA was found to be approximately 50%. The width of the form II DNA band did not increase significantly during the transfer process.

Hybridization. SV40 cRNA was synthesized essentially according to the method of Westphal (42). The reaction mixture contained 8 μg/ml of SV40 form I (covalently closed circular) DNA, 10 mM $MgCl_2$, 140 mM KCl, 33 mM Tris·HCl, pH 7.9, 1 mM dithiothreitol, 0.26 mM ATP, GTP, and CTP, 0.02 mM [^{32}P]UTP (specific activity: 90–120 Ci/mmol; New England Nuclear), 10% (vol/vol) glycerol, 124 μg/ml of bovine serum albumin, 0.1 mM EDTA, and 40 μg/ml of *Escherichia coli* RNA polymerase holoenzyme. Synthesis was carried out at 37° for 50 min. Electrophoretically purified DNase (Sigma) was added to a final concentration of 20 μg/ml and the incubation at 37° was continued for 10 min. The reaction mixture was extracted twice with phenol and the phenol was removed by several ether extractions. The SV40 [^{32}P]cRNA was separated from lower molecular weight compounds by chromatography on Sephadex G-75, collected by ethanol precipitation, and redissolved in a small volume of 2 × SSC. The specific activity of the cRNA product was generally 1 to 2 × 10^8 cpm/μg.

Hybridization of cRNA to the DNA immobilized on the membrane filters was carried out according to the method of Gillespie and Spiegelman (40). The hybridization solution contained 0.02–0.1 μg/ml of SV40 [^{32}P]cRNA and 200 μg/ml of carrier yeast tRNA (Calbiochem) in 2 × SSC. The filters were incubated in hybridization solution for 16–36 hr at 67°, then washed with 2 × SSC, treated with pancreatic RNase (20 μg/ml in 2 × SSC at 37° for 1 hr), and washed again with 2 × SSC. After drying, the filters were placed in close contact with a sheet of Kodak x-ray film (RP/S-54) for an appropriate time (12 hr to 2 weeks).

RESULTS

Experimental Approach. The rationale of our experiments is diagrammed in Fig. 1. Transformed cell DNA, containing a linearly inserted segment (or segments) of viral DNA, is digested with a sequence-specific restriction endonuclease. The fragments produced by the digestion are fractionated according to size by electrophoresis on an agarose gel, and the fragments containing viral DNA sequences are located by hybridization with ^{32}P-labeled SV40 cRNA after transfer of the DNA to a membrane filter (29, 41). The sizes of the fragments containing viral sequences are determined by the spacing of the restriction sites within and flanking the viral DNA. The spacing of the sites depends on the position of the joints between viral and host DNAs formed during integration; two transformed cell lines whose host or viral DNAs were broken at different points by the integration event will yield different sets of fragments containing viral

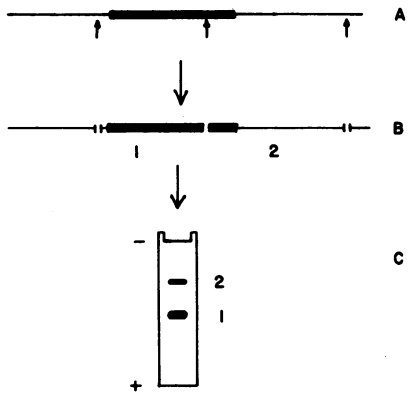

FIG. 1. A schematic representation of restriction enzyme analysis of integrated viral DNA. (A) Transformed cell DNA is depicted as containing segments of viral DNA (heavy line) integrated into host DNA (light line). The viral DNA is flanked by restriction sites (arrows) and in this example a site is present in the viral segment as well. (B) Digestion with a restriction endonuclease produces fragments of DNA, two of which (1 and 2) contain viral sequences. (C) After electrophoresis, transfer of the DNA to a membrane filter, and hybridization to ^{32}P-labeled viral RNA, autoradiography allows the detection of bands of DNA containing viral sequences. The positions of the bands reflect the arrangement of restriction sites in the original DNA.

DNA. This method can clearly be extended to locate fragments containing specific portions of the SV40 genome by using as a probe labeled RNA synthesized with purified viral restriction fragments as templates.

Sensitivity of the Method. In SV40-transformed cells the ratio of integrated viral DNA sequences to cell DNA sequences is on the order of 10^{-6}. A reconstruction experiment was carried out to determine whether the method described in *Materials and Methods* was sufficiently sensitive to reliably detect SV40 sequences at this level in a restriction enzyme digest of cell DNA. BALB/c 3T3 DNA was digested to completion with endonuclease R·*Hpa*II. Aliquots of this digest containing 10 μg of cell DNA were mixed with various quantities of SV40 form II DNA and electrophoresed in 1% agarose gels. Following electrophoresis the DNA was denatured *in situ*, transferred to a membrane filter, and incubated with high specific activity SV40 [^{32}P]cRNA under hybridization conditions as described in *Materials and Methods*. Fig. 2 shows an autoradiogram of the membrane filter. The band containing 10^{-5} μg of SV40 form II DNA is clearly visible, indicating that the method has the required sensitivity (one part in 10^6).

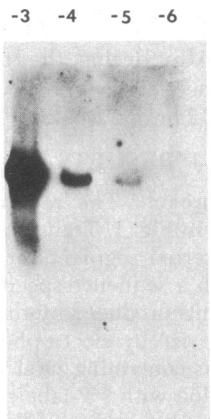

FIG. 2. Reconstruction experiment. Various quantities of SV40 form II DNA were mixed with 10 μg of a *Hpa*II digest of BALB/c 3T3 DNA, electrophoresed, transferred to a membrane filter, and hybridized to ^{32}P-SV40 cRNA as described in *Materials and Methods*. The figure shows an autoradiograph of the membrane filter developed after a 6-day exposure. The lanes marked −3, −4, −5, and −6 contained 10^{-3} μg, 10^{-4} μg, 10^{-5} μg, and 10^{-6} μg of SV40 DNA, respectively.

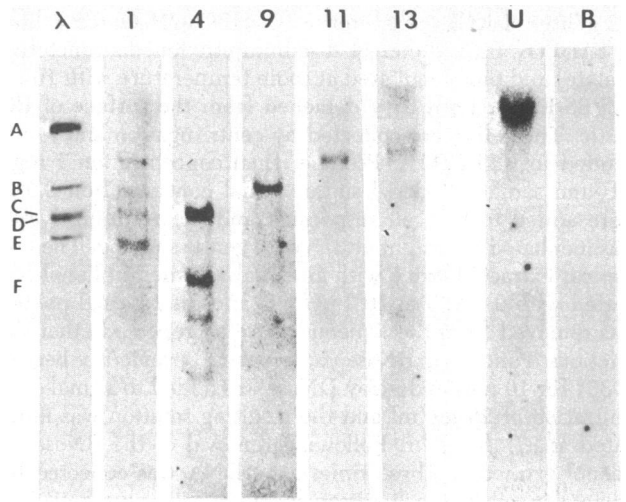

FIG. 3. Endonuclease R·*Hpa*II digests of SV40-transformed cell DNAs. Transformed cell DNAs (10–20 μg) were digested to completion with endonuclease R·*Hpa*II and electrophoresed in 1% agarose gels. After denaturation, transfer of the DNA to membrane filters, and hybridization, the filters were autoradiographed (see *Materials and Methods*). Lanes marked 1, 4, 9, 11, and 13 were loaded with digested DNA prepared from SV40-transformed lines SVB201, 204, 209, 211, and 213, respectively. Undigested SVB204 DNA was run in the lane marked "U" and BALB/c 3T3 DNA digested by endonuclease R·*Hpa*II was run in the lane marked "B".

It is expected that for a given transformed line, difference in the relative densities of the bands will reflect differences in the SV40 DNA content of the bands (see Figs. 1 and 2). However, a variety of factors, such as differences in transfer or hybridization efficiency, or differences in the representation of specific portions of the SV40 genome in the ^{32}P-labeled viral RNA, may also influence band density. In the three cases tested (SVB201, 204, and 209) the band patterns were invariant over at least a 5-fold range of enzyme concentration. The lane marked "λ" contains the endonuclease R·*Eco*RI restriction fragments of ^{32}P-labeled λ DNA. The molecular weights of the λ fragments are: A, 13.7×10^6; B, 4.7×10^6; C, 3.7×10^6; D, 3.6×10^6; E, 3.0×10^6; F, 2.1×10^6 (48).

Restriction Enzyme Digestion of DNA from SV40-Transformed Cell Lines. DNA was isolated from five SV40-transformed cell lines obtained independently from the BALB/c 3T3 clone A31[7]. The DNAs were digested with either of the restriction enzymes endonuclease R·*Hpa*II or endonuclease R·*Bam*HI, both of which are known to cut SV40 DNA at single sites [*Hpa*II at SV40 map position 0.73, ref. 36; *Bam*HI at SV40 map position 0.16 (C. Mulder and R. Greene, personal communication)]. After digestion, the restriction fragments were electrophoresed through 1% agarose gels, transferred to a membrane filter, and hybridized with ^{32}P-labeled SV40 cRNA.

Fig. 3 and 4 show autoradiograms of filters containing *Hpa*II and *Bam*HI fragments, respectively. Each cell line yields a simple pattern of fragments containing SV40 DNA. Furthermore, the fragment pattern is different for each line studied. In some cases fragments produced by cleavage of two different transformed lines with one of the enzymes have similar mobilities (such as the single bands present in the *Hpa*II digest of SVB211 and SVB213, Fig. 3). However,

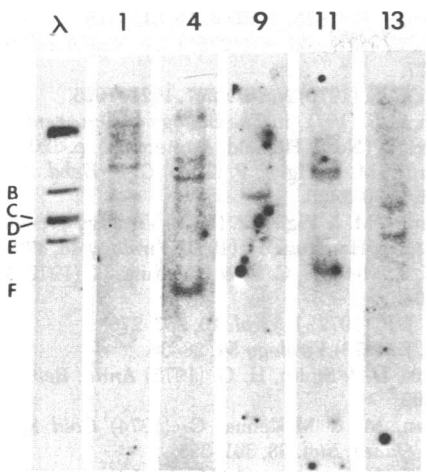

FIG. 4. Endonuclease R-*Bam*HI digests of transformed cell DNAs. SV40-transformed cell DNAs were digested to completion with endonuclease R-*Bam*HI and processed as described in the legend to Fig. 3.

examination of the fragments produced by the other enzyme (*Bam*HI, Fig. 4) makes it clear that the two lines are different.

In Fig. 3, autoradiograms of undigested SVB204 DNA and *Hpa*II-digested parental BALB/c 3T3 DNA are also shown. The distribution of SV40 sequences in the undigested DNA mimics the distribution of the total cellular DNA observed in similar gels stained with ethidium bromide. The distribution of SV40 sequences in undigested DNA from each of the other four transformed lines is indistinguishable from that of SVB204. The BALB/c 3T3 DNA is free of any detectable SV40 sequences.

DISCUSSION

Integration of SV40 DNA during transformation presumably requires some sort of recombination between the host and viral genomes. Probably the simplest model is that integration proceeds by a single reciprocal recombination which results in the linear insertion of the entire viral genome into the host genome (43). This model is consistent with the finding that all of the viral genetic information is preserved in some form in many SV40-transformed cell lines, since they yield wild-type SV40 virions following fusion with permissive cells (1–4). On the other hand, recent observations indicating that different regions of the SV40 genome are represented at different frequencies in the transformed line SVT2 suggest that more complicated models may be necessary, at least in some cases (44).

The question of whether or not integration proceeds via a site-specific mechanism is of considerable interest. Site-specificity with respect to the host or viral genomes, or both, could be mediated by a special recombination system which operates on specific nucleotide sequences in these DNAs (analogous to the integration systems of the bacteriophages λ, P2, P22, and mu). Alternatively, integration could occur by recombination between homologous regions of the host and viral genomes. In this case integration would be site-specific only to the extent that such regions of homology were limited in number and extent. Finally, integration may involve neither of these mechanisms and may belong to the class of poorly understood genetic events included under the term "illegitimate" recombination (45). In this case there is no expectation that integration would be site-specific. Some previous studies have suggested that in certain situations there may be some specificity to SV40 integration. Croce and Koprowski and coworkers have obtained evidence that the SV40 genetic information responsible for transformation of human cells may be integrated preferentially in chromosome number 7 (46). Recent studies on the substituted SV40 genomes which arise during high multiplicity passage have led to the suggestion that recombination between viral and host DNAs during productive infection of permissive cells may occur preferentially in a few small regions of the viral genome (47, 49). On the other hand, there is no evidence that recombination during productive infection occurs at specific sites in the host genome (49).

One approach to a better understanding of the molecular mechanism of integration is to define the structure of the integrated viral DNA. In this paper we have shown that information about the structure of integrated SV40 can be obtained by analysis of restriction endonuclease digests of transformed cell DNA. Both of the restriction enzymes used in this study cleave SV40 DNA at single sites. Thus, Fig. 1 represents the simplest of the possible outcomes of an experiment, i.e., the production of two fragments containing viral DNA. It should be pointed out, however, that a variety of circumstances could produce more complicated digest patterns. For example, more than two fragments containing viral DNA will be obtained if the integrated viral segment contains more than one cleavage site (e.g., because of a duplication) or the transformed cells contain more than one segment of integrated SV40 DNA. Only one fragment will be obtained if the integrated viral segment does not contain a cleavage site (e.g., because of a deletion). It is also possible that some fragments may escape detection because they contain too little SV40 DNA or because two different restriction fragments could coincidentally have the same mobility and thus be scored as a single fragment. These possible complications do not affect the conclusions of this paper, since the latter are based entirely upon a comparison of the restriction fragment patterns of different transformed cell DNAs and do not depend upon the details of the fragment pattern of any specific transformed cell DNA (see below).

Each of the SV40-transformed lines studied gave rise to a small number (four or fewer) of fragments containing SV40 DNA when digested with either the *Hpa*II or *Bam*HI enzymes. The relative simplicity of the observed band patterns indicates that the location of the integrated SV40 DNA is not randomized by excision and reintegration events during passage. If this were the case it would be expected that the band patterns would be very complex or undetectable. A similar conclusion has been reached by others (29).

The most important observation reported here is that the pattern of restriction fragments containing SV40 sequences is different for each of a series of SV40-transformed cell lines derived independently from the same parental line (BALB/c 3T3). This shows that the structure of the integrated SV40 DNA and/or its location in the host genome must be different in the various transformed lines. This in turn suggests that integration is not absolutely site-specific. We cannot exclude the possibility that there is specificity with respect to the recombinational site on *either* the viral genome *or* the host genome, but the data do appear to rule out specificity with respect to both sites simultaneously. This interpretation rests on the as yet untested assumption that rearrangements of the viral DNA (insertions, deletions, etc.) do not commonly occur either prior to or subsequent to the primary integration event. It should be noted that the data do

not exclude the possibility that integration occurs at specific nucleotide sequences if many such sequences are located at different sites on the host and/or viral genomes.

A variety of other questions concerning the structure of integrated SV40 DNA are amenable to analysis by more detailed application of the techniques described here. These include the questions of whether there is site-specificity limited to either the viral or the host recombination sites, whether the viral genome is generally integrated intact, whether a given transformed line contains one or more than one segment of integrated viral DNA, and whether the structure of the integrated viral DNA changes on passage. In addition, the sensitivity of this technique approaches that required to detect single copy mammalian genes, and may make it possible to partially purify by gel electrophoresis any such genes for which a suitable probe is available.

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