

Helper-Independent Transformation by Unintegrated Harvey Sarcoma Virus DNA

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We have studied the unintegrated infectious DNA of Harvey sarcoma virus (Ha-SV) and Moloney leukemia virus (Mo-MuLV). The source of infectious viral DNA was the Hirt supernatant fraction from cells acutely infected with Ha-SV and Mo-MuLV. To obtain a direct quantitative assay for infectious viral DNA, recipient mouse cells were first exposed to calcium phosphate-precipitated viral DNA and then treated with dimethyl sulfoxide. Infectivity was monitored by focus formation for Ha-SV and XC plaque formation for Mo-MuLV. The viral DNA titration pattern followed single-hit kinetics for both foci and plaques, indicating that a single molecule carried information for each function. Focus-forming and plaque-forming activity were present in different molecules, since these two biological activities could be separated from each other by agarose gel electrophoresis. The focus-forming molecule was linear DNA with a molecular weight of about 4×10^6 daltons. The focus-forming activity of the viral DNA was sensitive to *EcoRI* and resistant to *XhoI* restriction endonucleases, whereas the plaque-forming activity was resistant to *EcoRI* and sensitive to *XhoI*. The generation of helper-independent foci indicates that Ha-SV DNA can transform mouse cells in the absence of helper virus or its proteins.

Recent evidence has suggested that retroviruses replicate through an unintegrated double-stranded DNA intermediate which is then incorporated into the high-molecular-weight cellular DNA (35). Previous transfection studies of retroviral DNA (6, 31, 32) have shown that linear and circular unintegrated viral DNA from a replicating virus can be infectious for permissive cells, and it has been suggested that this infectious DNA represents the precursor to the integrated viral DNA.

The use of viral DNA from a replicating virus cannot easily determine if the infectious DNA is incorporated into the cell DNA of the transfected cell, since the spread of infectious virus through the transfected culture obscures events other than virus replication in the transfected cell. This problem can, however, be approached by using infectious DNA from a replication-defective sarcoma virus whose transforming activity can be measured in the absence of viral progeny. If transfection of sarcoma viral DNA were to induce transformation in the absence of helper virus, it would suggest that the unintegrated viral genome had become stably associated with the transfected cell and its descendants. Helper virus-independent transformation by sarcoma viral DNA would also answer the separate but related question of whether

helper viral proteins are required for cellular transformation by sarcoma viral DNA. This latter question cannot be resolved using whole virus, since even those sarcoma viral genomes which code for no known helper virus protein are always present in virions as pseudotypes which contain helper virus proteins.

Unfortunately, transfection with DNA from replication-defective viruses has in the past been quite cumbersome. In the only two previously published studies (16, 27), sarcoma viral DNA-induced transformation was seen irregularly, helper virus-independent transformation was observed only once, and transformed foci appeared only after 4 to 6 weeks in tissue culture. Recently, Stow and Wilkie (33) reported for herpes simplex virus DNA that brief treatment of cell monolayers with high concentrations of dimethyl sulfoxide (Me_2SO) after transfection of calcium phosphate-precipitated viral DNA (10) markedly increased the specific infectivity of the viral DNA. In the present study, we have adapted this technique to develop a direct quantitative assay for unintegrated sarcoma and helper viral DNA.

Using this assay, we have investigated the transforming activity of DNA from the Hirt supernatant fraction of cells acutely infected by a mixture of Harvey sarcoma virus (Ha-SV) and

Moloney murine leukemia virus (Mo-MuLV). Ha-SV, which is a recombinant between Mo-MuLV and endogenous rat sequences (29), does not synthesize any known Mo-MuLV protein (24). We show that the transforming activity of the Hirt DNA resides in the Ha-SV DNA and that the Ha-SV DNA can regularly transform permissive mouse cells independently of helper virus.

MATERIALS AND METHODS

Cells and viruses. The origins of BALB/c 3T3 (1) and NIH 3T3 (15) mouse embryo fibroblasts have been previously described. C1271, clone 4, cells were the gift of W. P. Parks and C. Chen. This nontransformed clonal line was derived from a mammary tumor of an RIII mouse. It forms a flat monolayer of cells and has been very useful for visualization of sarcoma virus-induced foci. It is also relatively resistant to treatment with high concentrations of Me₂SO. Supernatant fluid from these cells is negative for reverse transcriptase activity using Mg²⁺ or Mn²⁺ as cofactors. The NIH 3T3 cells chronically infected with Ha-SV and Mo-MuLV were described previously (23).

All cells were grown in Dulbecco-modified Eagle minimal essential medium with penicillin (10 U/ml) and streptomycin (100 µg/ml). This medium was supplemented with 10% calf serum (Colorado Serum Co.) for BALB/c 3T3 and NIH 3T3 cells. C1271 cells were grown in medium supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories). All cell lines were negative for mycoplasma by aerobic and anaerobic techniques (Flow Laboratories).

Virus was titrated on BALB/c 3T3, NIH 3T3, and C1271 cells. Mo-MuLV titers were determined by the XC plaque test (26), and Ha-SV titers were determined by focus formation (15). The chronically infected NIH 3T3 cells gave titers of 10⁶ to 10⁷ plaque-forming units and focus-forming units per ml on all three cell lines. The ratio of plaque-forming to focus-forming units varied from 2:1 to 4:1.

Hirt supernatant infectious viral DNA from acutely infected cells. Six to 10 plastic roller bottles (Corning) were seeded with 2 × 10⁷ NIH 3T3 cells. The following day, 125 ml of overnight supernatant fluid from the chronically infected (Ha-SV and Mo-MuLV) NIH 3T3 cells was placed in each bottle. In addition, 25 ml of fresh media and polybrene (4 µg/ml) (34) was added to each bottle at this time. Twelve to 15 h later (31), the cells were fractionated by the Hirt procedure (14) with one modification: instead of storing the precipitate in the cold overnight, the supernatant was separated from the pellet after 4 h at 4°C, since preliminary results indicated that overnight storage resulted in a significant decrease in yield of infectious viral DNA. The supernatant from the Hirt extract was then deproteinized twice with 1 volume of a 1:1 mixture of phenol and chloroform-isoamyl alcohol (24:1) and then ethanol precipitated. The precipitate was dissolved in 1 mM Tris, pH 7.5, and 1 mM EDTA and treated with RNase A at 100 µg/ml for 1 h at 37°C. The mixture was then treated for at least 1 h with proteinase K (Beckman) at 100 µg/ml, deproteinized by the phenol-chloroform method, ethanol precipitated, redissolved in 1 ml of 10 mM Tris, pH 8, and

dialyzed extensively against 10 mM Tris, pH 8. The Hirt supernatant DNA was then stored at 4°C.

For different preparations, the ratio of optical density at 260 nm (OD₂₆₀) to OD₂₈₀ varied between 1.85 and 2.00. Approximately 100 µg of DNA per roller bottle was found in the Hirt supernatant, which represented about 10% of the total cellular DNA. More than 90% of the Hirt supernatant DNA sedimented in a neutral sucrose gradient at less than 10S.

Isolation of cellular DNA from NIH 3T3 cells. The proteinase K method of Gross-Bellard et al. (12) was used, with minor modifications. This procedure resulted in DNA of average molecular weight greater than 4 × 10⁷ daltons and had an OD_{260:280} of 1.85 to 2.00. It was stored at 200 to 300 µg/ml (using 20 OD₂₆₀ units/mg of DNA) at 4°C in 10 mM NaCl-10 mM Tris (pH 8.0)-0.5 mM EDTA. This DNA from uninfected NIH 3T3 cells was used as carrier DNA for transfection experiments. As noted above, the infectious Hirt extracts were obtained from acute infection of this cell line.

Infectious DNA assay. Transfections were performed essentially by Stow and Wilkie's (33) modification of the method described by Graham and van der Eb (10), except for the pH of the *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline (HBS). The Hirt supernatant DNA was diluted to various concentrations in HBS (137 mM NaCl, 5 mM KCl, 7 mM Na₂HPO₄, 6 mM dextrose, and 20 mM HEPES, pH 6.85) containing 25 µg of NIH 3T3 DNA per ml as carrier DNA. The NIH Swiss mouse, from which the NIH 3T3 cells were derived, does not contain a complete ecotropic viral genome (5). The DNA was then precipitated by the addition of 2 M CaCl₂ to a final concentration of 125 mM and left at room temperature for 30 min before being added to the cultures. Initial experiments indicated that the use of unshredded NIH 3T3 DNA gave a single large precipitate and variable infectivity results. The NIH 3T3 DNA was therefore sheared 10 times with a 22-gauge needle attached to a 1-ml tuberculin syringe (18). These conditions resulted in the formation of a barely visible precipitate. (The same HBS solution may differ by as much as 0.15 pH unit with different pH meters. Optimal infectivity is obtained when the HBS is at the lowest pH which gives a slight turbidity after the addition of CaCl₂.) Portions (0.2 ml) of the DNA precipitate were then added to 35-mm petri dish cultures of C1271 cells which contained 1.5 ml of fresh medium (11). The dishes had been seeded the previous day at 2.6 × 10⁵ cells per dish so that the monolayer would be about 75% confluent at the time of addition of DNA. After 4 h of incubation, dishes were washed once with growth medium, and 0.75 ml of 25% Me₂SO in HBS was added to the cultures for 4 min at room temperature. The dishes were washed again with growth medium and then refed with 2 ml of growth medium. After an overnight incubation, the cells from each dish were trypsinized and seeded into three 60-mm petri dishes. Five days later, some dishes were treated with UV light for the XC assay. Others were held for the scoring of foci. Foci were usually first detected 5 to 6 days after DNA treatment; they were counted 3 to 4 days later. Departures from this standard procedure are noted in the text.

Preliminary experiments indicated that the infectivity of the Hirt supernatant viral DNA was similar on NIH 3T3, C3H, and C127I cells. C127I cells were chosen for this study because they are quite resistant to Me₂SO, and their flat background permits easy identification of sarcoma virus-induced foci. The C3H cells were quite difficult to propagate following Me₂SO treatment. Sarcoma-induced foci were difficult to score in the NIH 3T3 cells, but the cells were quite resistant to Me₂SO treatment.

Agarose gel electrophoresis. Gel electrophoresis of Hirt supernatant viral DNA was carried out as described by Sharp et al. (30) in cylindrical 0.7% agarose (Bio-Rad) gels (0.5 by 11 cm) containing 0.5 μg of ethidium bromide per ml for 18 h at 30 V. Unlabeled linear DNA fragments prepared by digestion of λ DNA (Bethesda Research Laboratories) with *EcoRI* restriction endonuclease (2) were used as marker DNAs for each gel. ³H-labeled simian virus 40 (SV40) DNA (forms I, II, and III), which was the gift of M. Martin, was run in parallel gels. The positions of the λ DNA bands were determined by UV fluorescence, and the gels were cut into 1.25-mm slices with a gel slicer. For transfection studies, two slices were combined, and the DNA was eluted from the gel as described (3, 6). Sheared NIH 3T3 DNA (10 μg) was then added as carrier, and the DNA was ethanol precipitated, dissolved in 0.2 ml of HBS, precipitated with CaCl₂, and transfected. Each slice of the gel containing the ³H-labeled SV40 DNA was placed in a scintillation vial and treated with 0.5 N KOH to dissolve the gel. The radioactivity was counted in a liquid scintillation counter (Beckman).

Sucrose gradient centrifugation of DNA. Hirt supernatant infectious viral DNA was centrifuged in a 5 to 20% neutral sucrose gradient in 100 mM NaCl-10 mM Tris (pH 7.2)-1 mM EDTA at 4°C for 150 min at 45,000 rpm in an SW50.1 rotor. Closed circular and linear ³H-labeled SV40 DNAs were centrifuged in a parallel marker gradient. The gradient was collected from the top into 23 equal fractions. Seven micrograms of sheared carrier DNA was added to each fraction, and each fraction was ethanol precipitated, redissolved in 0.2 ml of HBS, precipitated with CaCl₂, and transfected. The marker gradient was collected in a similar manner, and the radioactivity of each fraction was determined in a liquid scintillation counter.

Cesium chloride-ethidium bromide centrifugation of DNA. Hirt supernatant infectious viral DNA was centrifuged to equilibrium in a cesium chloride-ethidium bromide density gradient (17, 25) for 68 h at 30,000 rpm in an SW40 rotor. ³H-labeled colicin E1 factor DNA (form I and form III), which was the gift of M. Gellert, was centrifuged in the same gradient. The gradient was collected from the top, the ethidium bromide was extracted with isopropyl alcohol, and the DNA was dialyzed against 10 mM Tris, pH 8. After ethanol precipitation, the DNA was resuspended in 0.2 ml of HBS with carrier DNA, and the CaCl₂-precipitated DNA was transfected.

DNA:RNA hybridization. RNA was extracted as previously described (21). The single-stranded fractionated [³H]DNA probe which contains the rat sequences present in Ha-SV was prepared as previously described (28). Each 0.05-ml sample contained 20 mM Tris, pH 7.2, 600 mM NaCl, 0.17% sodium dodecyl

sulfate, 0.05 mM EDTA, 10 μg of yeast RNA, 1 to 250 μg of cell RNA, and approximately 2,000 trichloroacetic acid-insoluble cpm of ³H-labeled complementary DNA. After boiling, each sample was reacted under mineral oil at 66°C for 48 h. The samples were then digested with S₁ nuclease (4). The NIH 3T3 cell line chronically infected with Mo-MuLV and Ha-SV was used as the positive control and hybridized approximately 1,200 cpm. C127I RNA hybridized less than 200 cpm. At saturation, each Ha-SV DNA-transformed nonproducer C127I RNA hybridized at least 90% of the number of counts per minute of the positive control.

Restriction endonuclease digestion of Hirt supernatant infectious viral DNA. *EcoRI* (13) (Bethesda Research Laboratories) and *XhoI* (8) (New England Biolabs) restriction endonucleases were used under conditions described by these authors at a ratio of 4 U of enzyme per μg of Hirt supernatant DNA. Digestion by *EcoRI* was monitored by the complete digestion of added λ DNA. Digestion by *XhoI* was monitored by complete digestion of Ad2 DNA, which was the gift of J. Hauser. After treatment with the enzyme, the DNA was ethanol precipitated and resuspended in HBS, carrier DNA was added, and the DNA was precipitated with CaCl₂ and transfected into duplicate 35-mm dishes.

RESULTS

Me₂SO and carrier DNA enhance the efficiency of transfection. To determine the optimal conditions for the transfection assay, several parameters which might affect its sensitivity were tested. In the first experiment, the effect of Me₂SO and carrier DNA on the infectivity of Hirt supernatant viral DNA was examined. As seen in Table 1, brief treatment with 30% Me₂SO converted a marginally positive as-

TABLE 1. Effect of DMSO and carrier DNA on transfection of Hirt supernatant viral DNA^a

Carrier DNA (μg/ml)	Hirt viral DNA (μg/ml)	DMSO (%)	XC plaques ^b	Foci ^b
0	0	0	0, 0	0, 0
0	0	30	0, 0	0, 0
25	0	0	0, 0	0, 0
25	0	30	0, 0	0, 0
25	50	0	1, 1	0, 0
25	50	30	21, 16	10, 15
50	50	30	14, 12	5, 3
75	50	30	9, 10	2, 3
0	50	30	1, 0	2, 0

^a Transfections were carried out in duplicate 35-mm dishes as described in the text except that dishes received the carrier DNA, Hirt supernatant viral DNA, and Me₂SO as noted in the table. The 10 μg of Hirt DNA applied to each 35-mm dish represented 1.5% of the Hirt DNA preparation from six roller bottles. One of the three 60-mm dishes into which the cells from each 35-mm dish were divided was used for the XC test, and another was used to count foci.

^b Number per 60-mm dish.

say to one where each dish contained several areas of XC plaque-forming virus and focus-forming virus at about a 2:1 ratio. These results are consistent with those of Stow and Wilkie (33), who found that Me_2SO enhanced the infectivity of herpes simplex virus DNA, providing the treatment was not too toxic to the cells.

Graham and van der Eb (10), in their description of the calcium chloride technique for infectious adenovirus DNA, reported that when small amounts of purified viral DNA were used, the efficiency of transfection could be enhanced by the addition of carrier DNA (optimally at 25 to 30 $\mu\text{g}/\text{ml}$). Because most of the DNA in the Hirt supernatant is nonviral, relatively high concentrations of Hirt DNA would be required in the current studies. We therefore determined that, even at a high concentration of Hirt DNA, carrier DNA would enhance the infectivity. As seen in Table 1, even at 50 μg of Hirt supernatant viral DNA per ml, the addition of carrier DNA increased the number of plaques and foci. These data imply that the Hirt DNA does not substitute for carrier in the precipitated DNA, probably because more than 90% of the DNA in the Hirt supernatant is of very low molecular weight. Of the different concentrations of carrier DNA tested, 25 $\mu\text{g}/\text{ml}$ gave the best results.

Effect of pH of HBS on efficiency of transfection. Since Graham and van der Eb (10) found that the infectivity of adenoviral DNA was very dependent on the pH of the HBS, we also investigated this parameter. The HBS pH 6.8 was the lowest pH at which a visible precipitate formed, and HBS pH 6.8 and 6.9 gave the best infectivity for both foci and plaques (Fig. 1). This experiment was repeated twice, each time with similar results. These values are somewhat lower than the pH optimum of 7.0 to 7.1 reported by Graham and van der Eb. This difference is probably due at least in part to differences in the transfection conditions. Graham

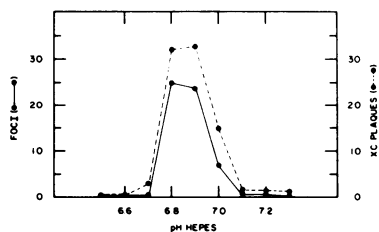


FIG. 1. Effect of pH of HBS on transfection of Hirt supernatant infectious viral DNA. Duplicate 35-mm dishes were exposed to 1% of a Hirt supernatant viral DNA preparation in HBS at the pH indicated and assayed for infectivity as described in the text. Foci (●—●) and XC plaques (●—●) are expressed as the number per 35-mm dish.

and van der Eb exposed the cultures to the precipitated DNA for 45 min before adding media, whereas here, the precipitated DNA was added to cultures that already contained media. Following the addition of CaCl_2 , the pH of the precipitated DNA was approximately 0.15 pH unit less than that of the HBS. These lower pH's will tend to be more toxic to cells when added to them in the absence of media.

Titration of infectious Hirt supernatant viral DNA. Having determined the optimal Me_2SO , carrier DNA, and HBS pH conditions, a titration of the Hirt supernatant viral DNA was carried out, using twofold serial dilutions. As shown in Fig. 2, both the plaques and the foci gave approximately one-hit kinetics (1.0 hit for plaques and 1.1 hit for foci), suggesting that each XC plaque and focus was the result of a single infectious molecule. Infectious virus did not appear in the culture fluid until more than 24 h after transfection; 1 ml of fluid from a dish which received a 10^{-2} dilution of Hirt viral DNA was found to contain no plaques or foci 24 h after transfection and only 1 plaque-forming unit and 1 focus-forming unit 48 h after transfection.

The infectious material in the Hirt supernatant was DNA, since treatment of the preparation with DNase prior to transfection abolished all infectivity. The transfected XC plaque-forming virus was presumptively identified as Mo-MuLV on the basis of host range studies; it formed XC plaques in equal titer on BALB/c 3T3 and NIH 3T3 cells and was therefore NB tropic, in contrast to known endogenous viruses

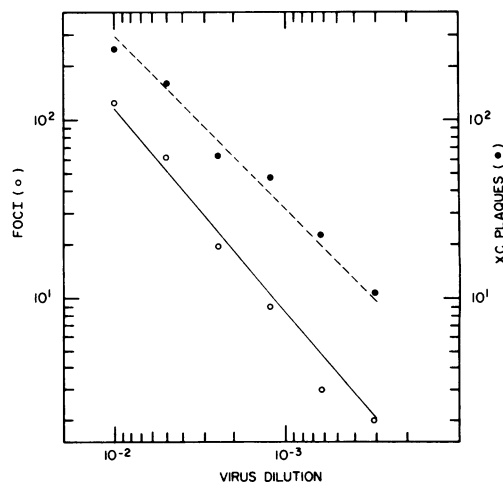


FIG. 2. Titration of Hirt supernatant infectious viral DNA. Duplicate 35-mm dishes were used for each dilution of Hirt viral DNA and assayed for infectivity as described in the text. Foci (○) and plaques (●) are expressed as the number per 35-mm dish.

(19). The focus-forming activity was due to a virus, since filtrates of media from transfected plates induced foci of transformed cells on recipient cells. This virus was identified as Ha-SV by nucleic acid hybridization of viral RNA to the rat-specific sequences present in Ha-SV (data not shown).

Separation of Mo-MuLV DNA from Ha-SV DNA. Although the foregoing experiment indicated that the XC plaque-forming virus was Mo-MuLV and the focus-forming virus was Ha-SV, foci were found only in conjunction with replicating Mo-MuLV. To establish that Ha-SV DNA could transform cells independently of helper virus, the Ha-SV DNA had to be shown to be capable of inducing transformation in the absence of infectious Mo-MuLV DNA. Since previous studies had shown that Ha-SV had a lower molecular weight than Mo-MuLV RNA (22, 23) and that more than 95% of the infectious Mo-MuLV DNA was a linear double-stranded DNA twice the molecular weight of one subunit of Mo-MuLV virion RNA (31, 32), it seemed likely that infectious Ha-SV DNA would also be a linear DNA twice the molecular weight of Ha-SV RNA and that the two infectious DNAs should therefore be separable on the basis of size.

Velocity centrifugation of the Hirt supernatant DNA in a neutral sucrose gradient failed to separate the focus-forming activity from the plaque-forming activity. To achieve a more complete separation between the Ha-SV DNA and Mo-MuLV DNA, agarose gel electrophoresis of the Hirt supernatant viral DNA was carried out with internal markers of λ DNA. ^3H -labeled SV40 DNA (forms I, II, and III) were run in control gels. Transfection of the Hirt DNA eluted from the gel slices showed complete recovery of input infectious DNA. The infectivity of the two viral DNAs each migrated as a single sharp peak with no overlap between them (Fig. 3). Approximately twice as many Mo-MuLV DNA-induced XC plaques as Ha-SV DNA-induced foci were present in the gel. This ratio is very similar to the biological ratio of helper virus to sarcoma virus used to infect the cells from which the Hirt supernatant extract was prepared. In agreement with the data of Smotkin et al. (32), the Mo-MuLV DNA had the migration rate of a linear double-stranded DNA of approximately 6×10^6 daltons. The Ha-SV DNA had the migration rate of a linear double-stranded DNA of approximately 4×10^6 daltons, as would be predicted from its viral RNA.

Biological and biochemical criteria were used to confirm that the transformed foci of fractions 21, 22, and 23 were Ha-SV nonproducer foci.

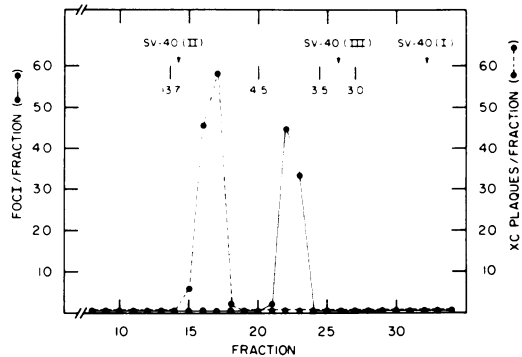


FIG. 3. Agarose gel electrophoresis of Hirt supernatant infectious viral DNA. Electrophoresis and the infectivity assay were as described in the text. Foci (●—●) and plaques (●----●) are expressed as the number per fraction. Molecular weights of linear *EcoRI*-restricted DNA fragments are $\times 10^{-6}$ daltons. The form I, II, and III markers are of closed circular, open circular, and linear SV40 DNA, respectively.

Cells from 10 foci were isolated with cloning cylinders and propagated. The media from these cells remained negative for virus by XC plaque test and by reverse transcriptase assay and failed to induce foci of transformed cells on C127I or BALB/c 3T3 cells. However, following superinfection of each culture with Mo-MuLV, media from these cultures readily induced foci in recipient cells. In addition, nucleic acid hybridization of the cellular RNA to a probe specific for the rat sequences present in Ha-SV indicated that these cells contained all the rat sequences present in Ha-SV.

To confirm that infectivity was not due to closed circular molecules, Hirt supernatant viral DNA was sedimented to equilibrium in a CsCl-ethidium bromide density gradient to separate closed circular DNA from linear or nicked circular DNA (25) (Fig. 4). For each viral DNA, a single superimposable peak of infectivity was found. These fractions coincided with the non-covalently closed ^3H -labeled colicin E1 DNA included in the gradient. These results confirmed that less than 5% of the infectious Mo-MuLV and Ha-SV DNA was covalently closed.

Sensitivity of infectious Ha-SV and Mo-MuLV DNA to restriction endonucleases. As noted earlier, Ha-SV represents a recombinant between Mo-MuLV and endogenous rat sequences. Since Ha-SV and Mo-MuLV share some, but not all, of their nucleic acid sequences, it was of interest to compare the sensitivity of the two infectious viral DNA genomes to digestion by restriction endonuclease. We have found that *EcoRI* destroyed the focus-forming activity, whereas more than 50% of the XC plaque-form-

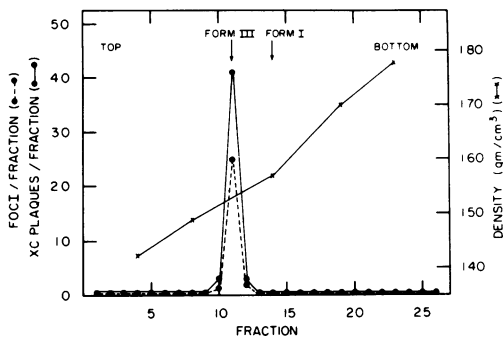


FIG. 4. Cesium chloride-ethidium bromide equilibrium density centrifugation. Centrifugation and the infectivity assay were as described in the text. Foci (●-●) and plaques (○-○) are expressed as the number per fraction. The form I and form III markers are of closed circular and linear ^3H -labeled colicin factor E1 DNA, respectively.

ing activity was resistant (resistance of Mo-MuLV DNA to *EcoRI* had been noted previously by Smotkin et al. [31]). By contrast, more than 50% of the focus-forming activity was resistant to *XhoI*, whereas *XhoI* abolished the XC plaque-forming activity. Use of this latter enzyme therefore represents another approach to the generation of Ha-SV DNA-transformed nonproducer cells. These data suggest that the *EcoRI* site in Ha-SV DNA is located in the rat sequences and that the *XhoI* site in Mo-MuLV DNA is located in the portion of the genome not present in Ha-SV. T. M. Goldfarb and R. A. Weinberg (personal communication) have recently found that Ha-SV DNA is cleaved once by *EcoRI* and not cleaved by *XhoI*, whereas Mo-MuLV DNA is cleaved once by *XhoI*; we have confirmed their results (D. R. Lowy and M. A. Israel, unpublished data). Therefore, cutting the Ha-SV DNA at its unique *EcoRI* site abolishes its transforming activity.

DISCUSSION

In this study, unintegrated linear Ha-SV DNA induced foci with single-hit kinetics in a direct quantitative transfection assay of the Hirt supernatant DNA obtained from cells acutely infected with Ha-SV and Mo-MuLV. The separation of infectious Ha-SV DNA from infectious Mo-MuLV DNA by agarose gel electrophoresis demonstrated directly that the DNA molecules which induced plaques were different from those which induced foci. The generation of helper-independent Ha-SV DNA-transformed cells implied that the unintegrated infectious Ha-SV DNA molecule can reproducibly associate itself in a stable manner within the transfected cell and can be biologically active in the transfected

cell's descendants in the absence of infectious virus. In the gel electrophoresis, the ratio of Mo-MuLV DNA-induced plaques to Ha-SV DNA-induced foci is approximately 2:1, which is very similar to the input ratio of leukemia to sarcoma virus in the cells from which the Hirt supernatant extract was prepared. These results suggest that the two viral DNAs register as successful transfectants with similar efficiency.

Although it is likely that the sarcoma viral DNA has been incorporated into the high-molecular-weight DNA of the transfected cell and maintained in this location in the descendants of that cell, further analysis of the transformed nonproducer cells will be required to establish this point. Recent data (3) suggest that integration is site specific in cells chronically infected with an avian retrovirus; it may be informative to compare the integration sites of cells transformed by transfected Ha-SV DNA with the integration sites of Ha-SV DNA in cells transformed by pseudotypes of Ha-SV.

Although these observations indicate that unintegrated Ha-SV DNA devoid of helper viral proteins can induce transformation, it should be recognized that all mouse cells contain endogenous retroviral genes (7, 20). These experiments cannot exclude the participation of cellular mouse genes either in the transfecting DNA or the recipient cells in facilitating transformation. Ha-SV DNA transformation is not, however, limited to the system reported here, since Ha-SV DNA also transforms other mouse cells (NIH 3T3 and C3H) and with a heterologous carrier DNA (mink) (unpublished data).

The production of helper-independent transformed cells suggests that transfection of sarcoma viral DNA may provide an additional approach to the generation of sarcoma virus-transformed nonproducer cells. The present study indicates the feasibility of separating helper virus DNA from sarcoma virus DNA by two different techniques: one on the basis of size differences, the other through the use of a restriction endonuclease which would inactivate the helper virus DNA without affecting the infectivity of the sarcoma virus DNA. This latter approach might be especially useful for those sarcoma viruses which have a high ratio of helper virus to sarcoma virus.

Since unintegrated Ha-SV DNA can induce transformation in the absence of helper virus, it may be possible to determine if the transformation event requires the entire sarcoma viral genome or if only a portion of the viral genome may be sufficient for the induction of transformation. Studies of two DNA viruses, adenovirus and SV40, have already shown that specific subgenomic fragments can transform cells (9),

and it would be of great interest to determine if this finding can be extended to include the sarcoma viruses. Experiments utilizing deletion mutants of Rous sarcoma virus which are defective for transformation have shown that nucleic acid sequences (the "src" gene) located near the 3' end of the viral RNA are required for cellular transformation (36), but it has not yet been established if these sequences by themselves can induce transformation. Studies of the biological activity of subgenomic portions of sarcoma virus nucleic acids may provide further insight into this problem.

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