Infectivity of Proviral DNA from Avian Sarcoma Virus-Transformed Mammalian Cells

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The number of Rous viral genomes in the cellular DNA from two subclones (RS2/3, RS2/6) derived from the same clone of hamster BHK-21 cells transformed by Rous sarcoma virus was determined by hybridization with viral complementary DNA made *in vitro*, and the capacity of the cellular DNA to infect (transfect) chicken embryo fibroblasts was compared before and after shearing this DNA to about the size of the provirus (6×10^6 to 7×10^6 daltons). The two subclones differed widely both in their capacity to give rise to virus (inducibility) after fusion with chicken embryo fibroblasts and in level of expression of viral proteins. It was shown that cells of both subclones contain a single copy of Rous DNA and yield infectious DNA. However, whereas transfection of chicken embryo fibroblasts was successful with both unsheared ($\geq 18 \times 10^6$ daltons) and sheared DNA from the most inducible subclone (RS2/3 subclone), which also expresses viral proteins to an appreciable amount, transfection with DNA from the least inducible subclone (RS2/6 subclone), in which viral proteins are not expressed, succeeded only with sheared DNA. It was then about as successful as with sheared or unsheared RS2/3 DNA. The lack of infectivity of unsheared RS2/6 DNA may be explained by the hypothesis proposed by Cooper and Temin (G. M. Cooper and H. T. Temin, *J. Virol.* 17:422-430, 1976) to explain the lack of infectivity of DNA from certain chicken cells producing spontaneously low amounts of RAV-0 and resistant to exogenous RAV-0 infection, that is, that the viral genome (proviral DNA) is linked to a *cis*-acting control element which blocks its expression. This linkage might originate, in RS2/6 cells, from translocation of cellular DNA containing the single proviral copy.

Mammalian cells transformed by avian sarcoma viruses are generally nonpermissive (see review, ref. 28), although some lines of transformed rodent cells shed low amounts of virus (23, 27). In a few nonpermissive (V^-) transformed cell lines, the viral genome that persists in the form of proviral DNA integrated with cellular DNA has been shown to be defective (20, 30). However, in most V^- lines studied, the viral genome appears to persist as a nondefective provirus, as is suggested by recovery of the transforming virus after either fusion of the V^- cells with permissive chicken cells (28) or transfection of the latter cells with DNA from V^- cells (14). Moreover, transfection succeeds only with DNA of over 6×10^6 daltons, that is, the size of the entire viral genome (14).

V^- mammalian cells from which virus can be recovered (virogenic cells) generally express some viral antigens and contain viral RNAs (10, 28). However, they display a number of differences with virus-producing (V^+) chicken cells which may each account for nonpermissiveness: (i) they contain much less viral RNA (5, 8); (ii)

some of the viral RNAs can be missing (N. Quintrell, M. Bishop, and N. Varmus, cited in ref. 10) or transported improperly from the nucleus to the cytoplasm (9); (iii) the cleavage of the precursor polyprotein of the viral p (or *gag*) protein of the *gs* antigenic complex may be defective (10, 22). In addition, V^- virogenic cells display a wide variability in levels of transcription of the proviral DNA into viral RNAs (8, 9) and of expression of viral proteins (1, 10), and levels of viral RNAs and proteins appear to parallel the level of virus induction after fusion with chicken cells (8, 32).

The parallelism between the levels of expression of viral proteins and of virus induction has been observed (32), in the case of p proteins of the antigenic *gs* complex, on a series of subclones isolated by one of us from a single clone (RS2) of hamster BHK-21 cells transformed *in vitro* by the Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV-D). Differences in levels of inducibility attaining 1,000-fold (as measured by the capacity of heterokaryons of RS2 cells and chicken embryo fibroblasts [CEF] to register as

infective centers) were scored between the most inducible subclones and the least inducible one (RS2/6); whereas an appreciable level of expression of viral p (*gag*) proteins and also gp85 (1) was found in the former subclones, no viral proteins could be detected in the latter one. Subsequent, unpublished investigations have further revealed that RS2/6 cells contain, on the average, only one to two copies of viral RNA (of undetermined size), against 10 to 20 copies for the most highly inducible RS2 subclone (RS2/3) (A. Weydert, manuscript in preparation). Hence, the low level of inducibility and the lack of expression of viral proteins in RS2/6 cells may both be related, at least in part, to a blockage of transcription of the proviral DNA into viral RNAs.

A hypothetical model consistent with this blockage is that proviral DNA may be present as a single copy in RS2 cells and might have become inserted by translocation in the vicinity of a *cis*-acting control element of the cellular genome in RS2/6 cells. This model, which was proposed initially by Cooper and Temin (7) to account for the inefficient expression of RAV-0 in certain chicken cells and the lack of infectivity of proviral DNA from these cells in transfection experiments, implies three predictions: (i) there should be no more than one proviral DNA copy in the genome of RS2 cells (or two tandemly inserted copies); (ii) transfection of CEF with large-size cellular DNA should be successful with DNA from RS2/3 cells but not with DNA from RS2/6 cells; and (iii) transfection with DNA from RS2/6 cells should be successful, and as efficient as transfection with DNA from RS2/3 cells, after the DNA is sheared to about the proviral genome size (6×10^6 to 7×10^6 daltons), thus removing the neighboring *cis*-control element from DNA fragments containing the complete proviral DNA.

We present here experimental data to support this hypothetical model.

MATERIALS AND METHODS

Cells and cell cultures. The origin and characteristics of the RS2/3 and RS2/6 subclones have been described (32). These cells were grown in Eagle minimal essential medium with a twofold concentration of amino acids and vitamins, plus 10% calf serum, 10% tryptose phosphate broth (Difco), and antibiotics (penicillin, streptomycin, and amphotericin B). Untransformed BHK-21 cells used as controls for DNA hybridization experiments were a clonal line (C13/15) isolated by L. Montagnier (Institut Pasteur, Paris). They were cultivated in the same medium as RS2 cells. XC cells, which were also used as controls in DNA hybridization studies, were obtained from the laboratory of J. P. Levy (Hôpital Cochin, Paris) and cultivated in the same medium. CEF were from the

Edinburgh Brown Leghorn strain and were lymphomatosis free but presumably *chf⁺ gs⁺* (1). Primary and secondary cultures were prepared as described earlier (12) in a standard medium composed of Eagle minimal essential medium with a twofold concentration of amino acids and vitamins, plus nonessential amino acids (also at a twofold concentration), 5% calf serum, 10% tryptose phosphate broth, and antibiotics. Secondary CEF cultures were also used for producing SR-RSV used for preparing viral RNA and complementary DNA (cDNA).

Virus. Virus used for preparing viral RNA and cDNA was a high-titer strain of SR-RSV-D obtained from D. Boettiger (Imperial Cancer Research Fund Laboratories, London) and passaged on Brown Leghorn CEF. This virus was concentrated from the infected culture medium by centrifugation in a Spinco 30 rotor (30,000 rpm, 60 min at 4°C) after removal of cell debris at lower speed. The virus pellet was suspended in NTE buffer (pH 7.4; 0.1 M NaCl–0.01 M Tris-hydrochloride [pH 7.4]–0.001 M EDTA) and purified, first by centrifugation onto a cushion of 60% sucrose overlaid with 20% sucrose (Spinco SW60 rotor, 30,000 rpm, 60 min, 4°C) and then by equilibrium density centrifugation using a gradient of 20 to 60% sucrose in NTE buffer (24) (Spinco SW60 rotor, 30,000 rpm, 5 h at 4°C).

Viral RNA. Viral 60 to 70S RNA was isolated as described by Smith et al. (25) and iodinated with ^{125}I using the method described by Prenskey et al. (21). The specific activity of the RNA was 10^7 cpm/ μg .

Viral cDNA. High-specific-activity radioactively labeled DNA complementary to SR-RSV-D 70S RNA was synthesized using detergent-disrupted virus (11). The incubation mixture contained 5 mg of purified virus at a concentration of 2 mg/ml in 0.05 M Tris-hydrochloride (pH 7.4), 0.01 M MgCl_2 , and 0.002 M dithiothreitol, plus 2×10^{-4} M each dATP, dCTP, and dGTP, 0.15×10^{-4} M [^3H]TTP (30 Ci/mmol), 0.025% (vol/vol) Nonidet P-40, and 100 μg of actinomycin D per ml. The incubation was carried out until the incorporation of radioactivity measured by trichloroacetic acid precipitation of 10- μl samples reached a plateau (6 h). The mixture was made 0.01 M EDTA and 0.1 M NaCl to stop the reaction, and DNA was purified by sodium dodecyl sulfate-phenol extraction and applied to a column (1 by 10 cm) of hydroxyapatite equilibrated with 0.04 M phosphate buffer (pH 6.8) at 55°C. The single- and double-stranded DNAs were eluted at the same temperature by increasing stepwise the phosphate buffer molarity by 0.05 M increments. Fractions containing single-stranded [^3H]DNA were pooled, dialyzed against 0.002 M EDTA, concentrated in the dialysis tube by adsorption against polyethylene glycol, and treated with 0.4 N NaOH for 30 min at 37°C. The cDNA had a specific activity of 25×10^6 cpm/ μg ; it was hydrolyzed to 97% by S1 nuclease. When a cDNA probe of lower specific activity (5,000 cpm/ μg), prepared under the same conditions, was hybridized with ^{125}I -labeled RSV RNA at various DNA:RNA ratios, 92% of the 70S viral RNA was protected against RNase digestion at a DNA:RNA ratio of 30. In an attempt to eliminate viral genome sequences excessively represented, the high-specific-activity cDNA was hybridized with 70S RNA at a

DNA:RNA ratio of 5, the hybridized DNA was separated by hydroxyapatite chromatography, and the RNA was hydrolyzed by NaOH as previously described. The cDNA sedimented at 6.5S in alkaline sucrose gradients.

Cellular DNA. Cellular DNA for either hybridization with viral cDNA or transfection of CEF was extracted and purified by a combination of the techniques of Marmur (19) and Berns and Thomas (2). The cells were suspended in NTE buffer (pH 8.0), lysed with sodium dodecyl sulfate at a final concentration of 0.4%, then digested overnight with 500 μ g of proteinase K per ml at room temperature. After two extractions with an equal volume of chloroform containing 4% isoamyl alcohol and another with an equal volume of NTE (pH 8.0)-saturated phenol until disappearance of the interphase, followed by extraction with ether to remove the remaining phenol, the nucleic acids were precipitated with cold ethanol. They were subsequently collected by winding on a glass rod, dissolved in 0.001 M EDTA-0.005 M Tris-hydrochloride (pH 8.0)-0.001 M NaCl, and treated, first with 100 μ g of pancreatic RNase (Worthington) per ml for 60 min at 37°C, then with 50 μ g of proteinase K per ml for another 60 min at 37°C. The solution was then made 0.1 M NaCl, 0.01 M EDTA, and 0.01 M Tris-hydrochloride (pH 8.0), extracted as described above with chloroform and NTE-saturated phenol, and then dialyzed extensively against 0.1 $\times$ SSC (1 $\times$ SSC: 0.15 M NaCl-0.015 M sodium citrate). The purified DNA was roughly quantitated by its UV absorption (absorbance at 260 nm = 1 for 41.5 μ g of DNA per ml). For hybridization with viral cDNA, purified cellular DNA was fragmented by limited depurination followed by alkaline hydrolysis (18) to obtain DNA fragments sedimenting at 6S in alkaline sucrose gradients. For transfection of CEF, purified cellular DNA was used either without further treatment (unsheared DNA) or after shearing by passing the solutions (100 to 200 μ g/ml in 0.1 $\times$ SSC) five times both ways (10 times in all) through needles of varying calibers attached to a 1-ml syringe. Sizes of unsheared and sheared DNA, as well as of depurinated DNA and viral cDNA, were all determined by sedimentation through 5 to 20% isokinetic alkaline sucrose gradients (16) (for 2 h at 50,000 rpm, Spinco SW60 rotor). Simian virus 40 [3 H]DNA (a generous gift from J. Feuntun, Institut de Recherches Scientifiques sur le Cancer, Villejuif) served as a standard. The S value of the linear single-stranded form (16S) of this DNA was adopted as the S value for DNA of 3×10^6 daltons. The molecular weights of other DNAs were calculated from their S values, using the Studier relationship (26).

DNA-DNA hybridization and reassociation.

All reactions were performed at 68°C in 0.8 M NaCl and contained 0.05 M Tris-hydrochloride (pH 7.4), 0.1% sodium dodecyl sulfate, 0.001 μ g of [3 H]cDNA per ml, and depurinated alkaline-denatured cellular DNA. They were carried out with 40 μ l of the annealing mixture in 100- μ l sealed capillary tubes. A single cellular DNA concentration was used, and the incubation time varied. Renaturation was followed by using 14 C-labeled cellular DNA (2,500 cpm/ μ g). Samples were frozen and stored at -20°C at the end of the incubation, and the extents of hybridization and re-

naturation were analyzed by hydrolysis with single-strand-specific S1 nuclease (17).

Transfection of CEF with cellular DNA. Infectivity of unsheared and sheared DNA from RS2/3 and RS2/6 cells was assayed by transfection of secondary CEF cultures seeded the previous day in 5-cm dishes, using either the DEAE-dextran method of Cooper and Temin (6) in the first set of experiments, or the calcium phosphate method of Graham et al. (13) in the three subsequent sets. In experiments using the latter method, DNA dissolved in SSC was diluted to 10 to 20 μ g/ml in HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid)-buffered saline (pH 7.05), and CaCl_2 (2 M) was added to a final concentration of 0.125 M. After 20 to 30 min of incubation at room temperature, samples of 0.5 ml of the microcrystalline precipitate formed, either undiluted (for doses of 10 or 5 μ g) or diluted (by gentle pipetting) in HEPES-buffered saline (for doses of 1 or 3 μ g), were added directly into the medium of the CEF cultures. The cultures were subsequently incubated at 37°C for 4 h, and the medium was then replaced by standard minimal essential medium supplemented with 5% fetal calf serum. This medium was also used for cultures transfected by the DEAE-dextran method and was replaced 3 days later by standard medium without fetal calf serum. In all cases, the cultures were all examined microscopically for the presence of foci of Rous cells 1 week after transfection, and negative cultures (the great majority) were then subcultured individually by trypsinization into 10-cm dishes in standard medium containing 2 μ g of polybrene per ml, to favor reinfection of the subcultured cells. These cultures were examined after another week, and the assay was then considered terminated.

In agreement with earlier reports (7, 15), no difference in sensitivity between the DEAE-dextran method and the calcium phosphate method was observed. Results obtained by both methods have therefore been grouped.

RESULTS

Number of proviral DNA copies in RS2/3 and RS2/6 cells. The presence of the viral genome in rescuable form in RS2 cells has been demonstrated by virus induction by fusion of these cells with CEF (32). Transfection of CEF with chromosomal DNA from RS2/3 cells was also successful (Vigier and Montagnier, unpublished data). This suggests that proviral DNA is integrated with this DNA, as in other avian sarcoma virus-transformed mammalian cells (5, 30).

To estimate the number of proviral DNA copies integrated in RS2/3 and RS2/6 cells, hybridizations of SR-RSV-D cDNA with an excess of DNA from RS2/3 and RS2/6 cells were performed. The data were plotted using the conventional log C_0t abscissae, where C_0 is the concentration in moles per liter and t is the time in seconds (4). The reference for estimation of the number of viral genome copies was provided by

the reassociation kinetics of nonreiterated sequences of ^{14}C -labeled BHK-21 DNA. As a control for the number of copies of proviral DNA in RS2 cells, hybridizations of viral cDNA with an excess of DNA from XC cells were also performed. The viral cDNA could hybridize to a maximum of 80% with XC DNA and only 55% with both RS2/3 and RS2/6 DNAs (Fig. 1). This lower maximum of hybridization could result from the experimental conditions used for pre-hybridization of cDNA to viral RNA, leaving a cDNA probe with some sequences still over-represented. In this case, the excess of cellular DNA over cDNA used in the hybridization assays could be insufficient to drive the reaction to completion. On the other hand, nonreiterated sequences of BHK-21 cellular DNA present as single copies reassociated at a $C_{0t_{1/2}}$ value of about 1.3×10^3 mol/liter·s. The viral cDNA probe hybridized to XC DNA with a $C_{0t_{1/2}}$ of about 10^2 mol/liter·s and with RS2/3 DNA and RS2/6 DNA at the same value of $C_{0t_{1/2}}$ of about 1.6×10^3 mol/liter·s. These results indicate the presence of about 13 copies of the viral genome per XC cell and of 0.8 copy per RS2/3 or RS2/6 cell. Hence, one may conclude that both RS2/3 and RS2/6 cells have the same content of viral genome sequences and that this content is of the order of one copy per cell.

Size of unsheared and sheared RS2 DNA.

The molecular weights of sheared and unsheared DNAs were determined as described in Materials and Methods on samples of the preparations to be assayed, by velocity sedimentation

FIG. 1. Kinetics of hybridization of SR-RSV-D [^3H]cDNA to BHK-21, RS2/3, RS2/6, and XC cellular DNAs. Hybridization reactions were performed as described in the text. (Δ) Hybridization to BHK-21 DNA. ($\blacksquare$) Hybridization to RS2/3 DNA. ($\bullet$) Hybridization to RS2/6 DNA. ($\square$) Hybridization to XC DNA. ($\circ$) Reassociation of ^{14}C -labeled BHK-21 DNA. BHK-21, RS2/3, and RS2/6 DNA concentrations were 10 mg/ml, and XC DNA concentration was 2.5 mg/ml.

in alkaline sucrose. Linear single-stranded simian virus 40 [^3H]DNA (16S) was used as a reference. Sedimentation in alkaline sucrose was chosen for determining the DNA size because it yields a sharper peak for control simian virus 40 DNA than sedimentation in neutral sucrose. It might be objected that DNA containing single-strand nicks will yield smaller fragments on alkaline gradients. This source of error was presumably negligible, however, since DNA peaks obtained on alkaline gradients did not display a higher proportion of small molecules than DNA peaks obtained by sedimentation of the same preparations on neutral sucrose gradients (data not shown). Figure 2a shows that, before any intentional shearing, at the end of all the steps needed to isolate DNA, the molecules had molecular weights ranging mainly beyond 18×10^6 . Some DNA preparations also contained a small proportion of molecules of lower molecular weight; in these cases, the preparations to be tested were fractionated on preparative neutral sucrose gradients, in addition to the steps described for isolation of DNA, the upper half of the gradient was discarded, and the lower half was retained and extensively dialyzed against $0.1\times$ SSC.

To obtain sheared DNA with an average molecular weight of 6×10^6 to 7×10^6 , corresponding to DNA fragments of about the size of the viral genome, DNA solutions were passed as described through needles with diameters of 0.8, 0.9, and 1 mm. Shearing through a 1-mm needle yielded a nonhomogeneous mixture containing a high proportion of DNA larger than genome size (Fig. 2b), and shearing through a 0.8-mm needle yielded mainly DNA smaller than genome size (Fig. 2d), whereas a relatively homogeneous mixture of DNA molecules with the expected genome size was obtained with a needle diameter of 0.9 mm (Fig. 2c). Consequently, we only compared the infectivity of unsheared DNA and DNA sheared through a 0.9-mm-diameter needle.

Transfection of CEF with unsheared and sheared RS2 DNA. As can be seen from Table 1, transfection of secondary CEF cultures with unsheared ($\geq 18 \times 10^6$ daltons) or sheared (6×10^6 to 7×10^6 daltons) DNA from RS2/3 cells was successful for each DNA in two experiments out of two, and the percentage of positive cultures, although low, was comparable. On the other hand, four experiments out of four with unsheared DNA from RS2/6 cells remained negative, even though the number of assay cultures was much higher. In contrast, four parallel experiments out of four with sheared RS2/6 DNA were all positive, and the overall incidence of positive cultures was comparable to that ob-

FIG. 2. Sedimentation of unsheared or sheared RS2/3 DNA at alkaline pH. Samples of 50 μ l containing over 150 μ g of DNA, prepared as described in the text, and about 8,000 cpm of simian virus 40 [3 H]DNA were sedimented through linear alkaline sucrose gradients as described in the text. Gradient fractions of 150 μ l were collected dropwise through a needle hole punctured in the bottom of centrifuge tubes, and the radioactivity (●) and optical density (■) of each fraction were determined. (a) Unsheared DNA; (b) DNA sheared through a 1 by 38 mm needle; (c) DNA sheared through a 0.9 by 38 mm needle; (d) DNA sheared through a 0.8 by 38 mm needle. Underlined figures above full arrows are molecular weights, in kilodaltons, calculated from that of linear simian virus 40 DNA.

served with unsheared or sheared RS2/3 DNA. Comparison of the overall incidences of positive cultures, neglecting the doses of DNA used in the assays, was justified by the finding that, for the dose range studied (1 to 10 μ g per culture), no significant differences of incidence of positive cultures were scored with any of the DNAs.

On this same basis, the statistical significance of the difference between the mean number of positive cultures in the series with sheared and unsheared RS2/6 DNA (9/64 and 0/60) was calculated using Student's *t* test and found to be highly significant ($P < 0.01$). On the other hand, as expected, the differences between the mean numbers of positive cultures in the series with sheared RS2/6 DNA and sheared and unsheared RS2/3 DNA were not significant ($P > 0.05$).

DISCUSSION

The data presented support entirely the hypothetical model of a *cis*-acting cellular control element blocking transcription of the proviral

DNA in RS2/6, but not RS2/3, cells, since: (i) RS2/3 and RS2/6 cells appear to contain no more than one proviral copy per cell; (ii) CEF can be transfected with unsheared DNA of RS2/3 cells only; and (iii) CEF can be transfected with RS2/6 DNA sheared to about genome size as well as with sheared or unsheared RS2/3 DNA.

The finding that RS2 cells presumably contain only one copy of proviral DNA agrees with earlier data which showed (8, 31) that most of the avian sarcoma virus-transformed mammalian cells studied contained one to two copies of integrated proviral DNA. Varmus et al. (31) have also found that, in contrast with other avian sarcoma virus-transformed mammalian cell lines, the XC cell line contains about 20 copies of integrated proviral DNA. Our figure of 13 copies for these same cells is of the same order of magnitude. The difference from that of Varmus et al. might be due to differences in methods or in the cell lines.

TABLE 1. Assay of unsheared and sheared DNA from RS2/3 and RS2/6 cells^a

Donor DNA	Fraction of positive DNA preparations	Fraction of positive recipient cultures at DNA dose:				
		10 μ g	5 μ g	3 μ g	1 μ g	Total
RS2/3						
U	2/2		1/12		2/4	3/16
S	2/2	1/6	1/8	0/6		2/20
RS2/6						
U	0/4	0/10	0/32	0/6	0/12	0/60
S	4/4	2/16	3/28	3/12	1/8	9/64

^a Secondary CEF cultures were infected (transfected) with unsheared (U) or sheared (S) DNA from RS2/3 or RS2/6 cells, as described in the text, using either the DEAE-dextran method (for one preparation out of two for RS2/3 DNA and one out of four for RS2/6 DNA) or the calcium phosphate method (for other preparations). In the latter method, the DNA was added directly to the medium of cultures (three to four cultures per dose) seeded the previous day in 5-cm dishes, and the medium was renewed 4 h later. Transfected cultures were incubated for 3 days in standard medium supplemented with 5% fetal calf serum, then in standard medium, and were examined for the presence of Rous cells after 1 week. Negative cultures were then subcultured individually into 10-cm dishes, and these subcultures were examined after another week.

The finding that RS2 DNA hybridized with only 55% of the viral cDNA probe, against 80% for XC DNA, raises a problem, however. As previously mentioned, this low extent of hybridization may be due to the experimental conditions, but one may also argue that proviral DNA in RS2 cells, contrary to XC proviral DNA, might be defective and complemented in the *chf⁺ gs⁺* CEF used for transfection. This possibility is contradicted, in the case of RS2/3 cells, by the earlier finding (Vigier, unpublished data) that induction of SR-RSV-D can occur after fusion of these cells with *chf⁺ gs⁻* CEF. On the other hand, if RS2/6 proviral DNA is defective, transfection with sheared RS2/6 DNA should be less efficient than transfection with sheared RS2/3 DNA.

Moreover, earlier data of Svoboda et al. (29) suggest that transfection with XC cell DNA of the same CEF that were used in our experiments (Edinburgh, Brown Leghorn strain) is sometimes as inefficient as that with RS2 cell DNA. This raises the problem of the low infectivity of DNA from RS2 cells relative to that of DNA from V⁺ chicken cells (6). This may be due to a lower number of viral genomes in RS2 cells, but could also result from the integration of the viral genome with heterologous DNA. In favor of this

last possibility are the results of Svoboda et al. (29) showing that transfection with XC DNA may be unsuccessful with doses up to 150 μ g.

Inefficiency of transfection with RS2 DNA may also explain the absence of dose difference observed in transfection with amounts of DNA between 1 and 10 μ g. A similar absence of dose dependence was noted by Svoboda et al. (29) for doses of XC DNA ranging from 1.5 to 150 μ g. Another cause for inefficiency of transfection by RS2 DNA may be the virus used for transforming BHK-21 cells to RS2 cells. This is suggested by the earlier finding that transfection with DNA from CEF infected with a clonal derivative from the same virus (SR4 clone of SR-RSV-D) was barely more efficient than transfection with RS2 DNA and also apparently not dose dependent for comparable amounts of DNA (33).

On the other hand, the finding that infectivity of sheared RS2/3 DNA is comparable to infectivity of sheared RS2/6 DNA suggests that there is no major alteration of the viral genome in RS2/6 cells, relative to RS2/3 cells. Hence, RS2/3 and RS2/6 cells may differ only by the site of integration of the Rous genome. Results similar to ours, which confirm the Cooper-Temin model for the system for which it was proposed, have also been obtained recently with DNA from chicken cells producing spontaneously a low titer of RAV-0 and resistant to exogenous RAV-0 infection (Cooper, personal communication).

It may finally be recalled that, to account for the variability of the expression of the avian sarcoma virus genome in heterologous cells, it has been postulated (3) that these cells may lack a specific site of integration for the provirus leading to random integration into host cell DNA regions that are under different degrees of transcriptional activation.

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