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Mechanism of Transfection of Chicken Embryo Fibroblasts by Rous Sarcoma Virus DNA

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The mechanism of transfection by Rous sarcoma virus DNA was investigated by assaying DNA-mediated transformation under conditions which restricted secondary virus infection. Chicken embryo fibroblasts which were genetically resistant to virus infection as a result of the absence of receptors for virus penetration were also resistant to transformation by integrated or unintegrated Rous sarcoma virus DNA. In addition, DNA of replication-defective Bryan high-titer Rous sarcoma virus was noninfectious, and transformation by DNA of a temperature-sensitive DNA polymerase mutant was temperature sensitive. These results indicated that secondary virus infection was necessary for transformation by Rous sarcoma virus DNA. Since transformation was assayed by colony formation in soft agar, as well as by focus formation, the requirement for secondary virus infection was not an artifact of potential difficulty in detection of foci formed by division of single transformed cells. Therefore, it appeared that donor DNA did not stably transform recipient cells by direct integration. Instead, the results were consistent with the hypothesis that transfection of chicken embryo fibroblasts by Rous sarcoma virus DNA proceeded by transcription of donor DNA, formation of extracellular progeny virus, and secondary virus infection of sensitive cells.

Assays of the biological activity of virus DNAs by transfection have been used to study integrated and unintegrated DNAs of avian and mammalian retroviruses. However, the mechanism of transfection by retrovirus DNAs has not been established. Since this mechanism might be a significant consideration in interpretation of the biological activity of virus DNAs, we initiated the present study to investigate the mechanism of transfection of chicken embryo fibroblasts by DNA of Rous sarcoma virus (RSV).

Two general alternative pathways of transfection were considered: (i) donor RSV DNA might integrate directly into the genome of recipient cells to form a stably inherited DNA provirus or (ii) donor RSV DNA might serve as a template for RNA synthesis, possibly without integration of the donor DNA. In the second pathway progeny viral RNA would serve as a template for synthesis of RSV DNA which would subsequently integrate into cellular DNA. Synthesis and integration of RSV DNA might occur either within the cell which was the initial recipient for uptake and transcription of the donor DNA or via formation of extracellular progeny virus and secondary infection of other cells in the DNA-treated recipient cell culture.

To distinguish between these pathways, we investigated RSV DNA-mediated transformation of chicken embryo fibroblasts under conditions which restricted secondary virus infection. Transformation of recipient cells by RSV DNA was found to require formation of extracellular progeny virus and secondary infection of sensitive cells. Therefore, it appeared that donor RSV DNA did not stably transform recipient cells by direct integration. These results were thus consistent with the hypothesis that transfection of chicken embryo fibroblasts proceeded primarily by transcription of donor RSV DNA.

MATERIALS AND METHODS

Cells and viruses. Cells were grown in Temin modified Eagle minimal essential medium containing 20% tryptose phosphate broth (ET medium) and supplemented with calf and fetal bovine sera. Fertile chicken eggs were purchased from SPAFAS, Norwich, Conn. (C/E phenotype), from Hyline Farms, Dallas Center, Iowa (C/O and C/BDE phenotypes), and from H and N Farms, Redmond, Wash. (C/ACE phenotype). Chicken embryo fibroblasts were virus negative, chicken helper factor negative, and avian leukosis virus group-specific antigen negative. Fertile Orlopp turkey eggs were provided by the Wilmar Poultry Co., Wilmar, Minn. Turkey embryo fibroblasts were T/BD, virus negative, helper factor negative, and avian leu-

kosis virus group-specific antigen negative.

B77 avian sarcoma virus (subgroup C) (B77V-C), Schmidt-Ruppin RSV subgroup D (SR-RSV-D), and *LA335* (*pol*^{ts}), an early temperature-sensitive mutant of Prague RSV subgroup C which encodes a thermolabile DNA polymerase (15, 18, 29), were previously described (3). Bryan high-titer RSV (BH-RSV), which is defective for envelope glycoprotein synthesis (*env*$^-$) (22), was obtained as a Rous-associated virus-1 (RAV-1) pseudotype [BH-RSV(RAV-1)] from H. M. Temin, Madison, Wis.

Preparation of DNA. Total cellular DNA was extracted from RSV-infected chicken embryo fibroblasts 5 to 10 days after infection by a previously described modification (4) of the procedure of Marmur (17).

Unintegrated RSV DNA was extracted 3 days after infection of chicken embryo fibroblasts with SR-RSV-D (approximately 0.1 focus-forming unit [FFU]/cell). Cellular DNA was ^{3}H-labeled by culturing the cells for 24 h before DNA extraction in medium which contained 1 μCi of [^{3}H]thymidine per ml. Cells were harvested by trypsinization, suspended at a density of 5×10^6 cells/ml in 10 mM Tris-hydrochloride–10 mM EDTA (pH 7.4), and fractionated by the method of Hirt (12). Approximately 12% of the ^{3}H-labeled DNA was recovered in the Hirt supernatant fraction. The Hirt supernatant was digested with Pronase (250 μg/ml, 30 min, 37°C) and was extracted twice with chloroform-isoamyl alcohol (24:1). DNA was precipitated with ethanol overnight at −20°C. The precipitate was collected by centrifugation, dissolved in SSC (0.15 M NaCl-0.015 M sodium citrate, pH 7.0), digested with RNase A (100 μg/ml, 30 min, 37°C), digested with Pronase (250 μg/ml, 30 min, 37°C), and extracted six times with chloroform-isoamyl alcohol. NaCl was added to a final concentration of 0.3 M, and DNA was precipitated with ethanol overnight at −20°C. The precipitate was collected by centrifugation and was dissolved in sterile SSC. The final yield of Hirt supernatant DNA corresponded to approximately 3% of the initial ^{3}H-labeled cellular DNA.

Agarose gel electrophoresis. Hirt supernatant DNA was subjected to electrophoresis in 0.7% agarose gels to separate linear and circular forms of RSV DNA. Electrophoresis was performed as described by Sharp et al. (24), except that both the gels and the electrophoresis buffer contained 0.1 M NaCl and 4 μg of ethidium bromide per ml to optimize separation of circular and linear DNA forms (13). Covalently closed circular and linear DNAs of the plasmid pmDm691 (molecular weight, 6.2×10^6) (D. J. Finnegan, G. M. Rubin, and D. S. Hogness, in preparation) were used as markers. pmDm691 DNA was kindly provided by G. M. Rubin. The positions of infectious RSV DNAs were determined by transfection assays of DNA eluted from gel slices as described by Fritsch and Temin (7). The positions of marker DNAs in parallel gels were determined by UV fluorescence.

Transfection assay of RSV DNA. Cultures of chicken embryo fibroblasts were prepared and exposed to DNA by the calcium method of Graham and Van der Eb (10) as previously described (5). Recipient cells were incubated with DNA for 4 h, the medium was changed to ET medium supplemented with 2% fetal bovine serum, and the cells were incubated at 37°C. Media were changed at 3-day intervals, and foci of transformed cells were counted 5 to 7 days after exposure to DNA.

Colony formation in soft agar. Colony formation by transformed cells was assayed by a modification of the method of Graf (8). Feeder layers of mitomycin C-treated turkey embryo fibroblasts in 60-mm tissue culture dishes were overlaid with 3 ml of medium 199 supplemented with 20% tryptose phosphate broth, 0.06% sodium bicarbonate, 1% dimethyl sulfoxide, 5% calf serum, 1% heat-inactivated chicken serum, and 0.6% agar. DNA-treated chicken embryo fibroblasts were plated at a density of approximately 10^6 cells per dish in 4 ml of medium 199 supplemented as above but containing 0.36% agar. Cultures were incubated at 37°C, and colonies of transformed cells were counted 7 to 10 days after plating.

RESULTS

Transformation of sensitive and resistant chicken embryo fibroblasts by RSV DNA. Transfection assays of RSV DNA were previously quantitated by end point dilution of the donor DNA (4). However, by use of the calcium method of DNA treatment (10), transfection by RSV DNA can be quantitated by enumeration of foci on the original DNA-treated plates. The advantage of the calcium method in this respect appears to be that it is significantly less toxic to the recipient cells than the DEAE-dextran method used previously (4). Representative transfection assays are presented in Fig. 1A. The kinetics of transfection by RSV DNAs

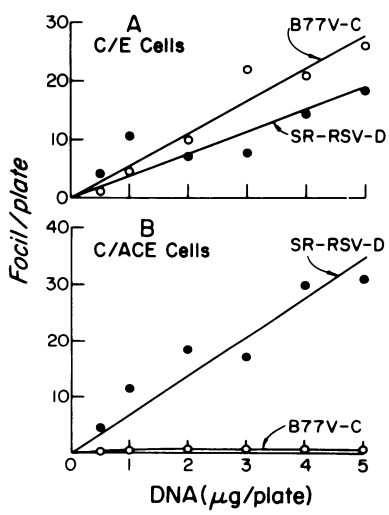

FIG. 1. *Focus assay of RSV DNA. Cultures of C/E or C/ACE chicken embryo fibroblasts were exposed to DNA extracted from B77V-C-infected cells (B77V-C DNA, ○) or from SR-RSV-D-infected cells (SR-RSV-D DNA, ●). Foci were counted 6 days after DNA treatment. Each point is the average of duplicate plates.*

were one hit, and the specific infectivities of RSV DNAs were approximately 5 FFU/μg (Fig. 1A). The specific infectivities of RSV DNAs assayed by focus formation were therefore similar to specific infectivities previously determined by end point dilution assays (approximately 10 infectious units/μg of DNA) (4).

The availability of a direct focus assay for RSV DNA enabled us to investigate transformation by RSV DNA in the absence of secondary virus infection. We therefore assayed the infectivity of RSV DNA on recipient cells which were genetically resistant to secondary virus infection as a result of the absence of receptors required for virus penetration (6, 21). B77V-C and SR-RSV-D DNAs had similar specific infectivities (5 and 4 FFU/μg of DNA, respectively) when assayed on C/E cells, which were sensitive to infection by both subgroup C and subgroup D RSV (Fig. 1A). However, B77V-C DNA was not infectious (<0.03 FFU/μg of DNA) when assayed on C/ACE cells, which were resistant to infection by subgroup C RSV (Fig. 1B). In contrast, the infectivity of SR-RSV-D DNA on C/ACE cells (Fig. 1B) was similar to its infectivity on C/E cells (Fig. 1A). Therefore, it appeared that recipient cells which were resistant to infection by extracellular RSV were also resistant to transfection by RSV DNA. Since subgroup-specific resistance to RSV infection appears to be expressed at the level of virus penetration (6, 21), these results indicated that focus formation by RSV DNA required formation of extracellular progeny virus and secondary infection of sensitive cells.

To test the possibility that secondary virus infection was only necessary for development of detectable foci under the conditions of transfection assays, we used colony formation in soft agar to assay transformation by RSV DNA. Since soft agar colonies are formed by division of single transformed cells, this assay eliminated the potential requirement for secondary virus infection in focus development.

Preliminary experiments were performed to determine the optimal time for transfer of DNA-treated recipient cells into suspension in soft agar. Recipient cells transferred into soft agar immediately after exposure to RSV DNA did not give rise to transformed cell colonies, but colonies were formed by cells transferred into soft agar 1 to 2 days after transfection. The failure of cells to form colonies in soft agar immediately after transfection by RSV DNA was consistent with the hypothesis that formation of progeny virus and secondary virus infection were needed for establishment of genetically stable transformation. The number of soft agar colonies formed by cells transferred 1 day after transfection ranged from 10 to 50% of the number of foci formed on parallel plates maintained under liquid medium, whereas the number of colonies formed by cells transferred 2 days after transfection equaled or exceeded the number of foci obtained. Therefore, recipient cells were routinely transferred into soft agar 2 days after transfection by RSV DNA.

B77V-C and SR-RSV-D DNAs were assayed on C/O, C/ACE, and C/BDE chicken embryo fibroblasts (Table 1). Some of the recipient cell cultures were maintained under liquid medium to assay focus formation. The rest of the recipient cell cultures were trypsinized 2 days after exposure to DNA and were plated in soft agar medium to assay colony formation. B77V-C DNA was infectious for C/O and C/BDE cells but not for C/ACE cells (resistant to infection by B77V-C). Reciprocally, SR-RSV-D DNA was infectious for C/O and C/ACE cells but not for C/BDE cells (resistant to infection by SR-RSV-D). With both DNAs, the results of soft agar colony assays agreed with the results of focus assays. Therefore, secondary virus infection appeared necessary for genetically stable transformation of recipient chicken cells by RSV DNA.

Transfection by DNAs of *LA335* (*pol*^{ts}) and BH-RSV (*env*$^{-}$). The role of secondary virus infection was further investigated in transfection experiments with DNAs of replication-defective mutant RSV. The mutants used were *LA335* (*pol*^{ts}), which has a temperature-sensitive mutation in the gene encoding the virion RNA-directed DNA polymerase (15, 18, 29), and BH-RSV (*env*$^{-}$), which has a deletion of the gene encoding the virion envelope glycoprotein (22).

DNA of *LA335*-infected cells was assayed for colony formation in soft agar at permissive

TABLE 1. *Assay of RSV DNA by colony formation in soft agar*[a]

DNA	Recipient cell phenotype	Foci/plate[b]	Colonies/plate[b]
B77V-C	C/O	24	50
	C/ACE	0	0
	C/BDE	10	48
SR-RSV-D	C/O	76	172
	C/ACE	64	122
	C/BDE	0	0

[a] Cultures of chicken embryo fibroblasts were exposed to 5 μg of B77V-C or SR-RSV-D DNA. Some cultures were maintained under liquid medium and foci were counted 7 days after DNA treatment. Other cultures were transferred into soft agar 2 days after DNA treatment and transformed cell colonies were counted 8 days later.

[b] Average of quadruplicate DNA-treated recipient cultures.

(35°C) and nonpermissive (41°C) temperatures. The infectivity of *LA335* (*pol*^{ts}) DNA was approximately 10-fold lower at 41 than 35°C (Table 2). In contrast, the infectivity of B77V-C (*pol*$^{+}$) DNA was threefold higher at 41 than 35°C (Table 2), possibly as a result of enhanced production of progeny virus at the higher temperature. The temperature sensitivity of transformation by *LA335* DNA indicated that DNA polymerase activity was required for transfection. The lower temperature sensitivity of transfection by *LA335* DNA (approximately 10-fold) as compared to the temperature sensitivity of virus infection by *LA335* (approximately 10^3-fold) (data not shown) may be due to continuous production of multiple progeny virus particles by the DNA-treated cells (see Fig. 3).

To test the infectivity of DNA of BH-RSV (*env*$^{-}$), we assayed transfection by DNA of cells infected with BH-RSV(RAV-1). This DNA was negative when assayed for transformation either by focus formation (<0.05 FFU/μg of DNA) or by colony formation in soft agar (<0.03 colony-forming unit/μg of DNA). However, BH-RSV(RAV-1) DNA was infectious (approximately 0.5 infectious unit/μg of DNA) when assayed for production of nontransforming virus by determination of sedimentable DNA polymerase activity in culture fluids (5). Therefore, BH-RSV (RAV-1)-infected cells appeared to contain infectious DNA of RAV-1 but not of BH-RSV (*env*$^{-}$). The lack of transformation by BH-RSV (*env*$^{-}$) DNA further indicated that secondary virus infection was required for RSV DNA-mediated transformation.

A control experiment was performed to determine whether BH-RSV-transformed nonproducer cells formed soft agar colonies under the conditions used for transfection assays. Cultures of chicken embryo fibroblasts were treated with salmon sperm DNA and were then infected with serial dilutions of a BH-RSV(RAV-1) stock which titered approximately 2 × 10^5 FFU/ml in standard focus assays. Two days after infection, the cells were trypsinized and plated in soft agar. The efficiency of colony formation corresponded to approximately 5 × 10^5 colony-forming units/ml of the original virus stock and was thus similar to the titer determined by focus assay. Twelve transformed cell colonies from plates which had been exposed to a 10^{-4} dilution of virus were picked and tested for production of infectious progeny RSV. Nine of the 12 colonies failed to yield infectious progeny virus, indicating that transformed nonproducer cells were capable of forming soft agar colonies under transfection assay conditions.

TABLE 2. *Transfection by LA335 (pol*^{ts}*) DNA*[a]

DNA	Colonies/plate[b]	
	35°C	41°C
B77V-C (*pol*$^{+}$)	31	92
LA335 (*pol*^{ts})	5.3	0.4

[a] Cultures of C/O chicken embryo fibroblasts were exposed to 5 μg of *LA335* (*pol*^{ts}) or B77V-C (*pol*$^{+}$) DNA and were incubated at either 35 or 41°C. The cells were transferred into soft agar 2 days after DNA treatment.

[b] Average of three plates treated with B77V-C (*pol*$^{+}$) DNA or 27 plates treated with *LA335* (*pol*^{ts}) DNA.

Transfection by unintegrated RSV DNA. Since total cellular DNA of RSV-infected cells did not appear to stably transform recipient cells by direct integration, it was of interest to investigate transfection by newly synthesized unintegrated RSV DNA, which is the probable precursor to integrated proviral DNA in virus-infected cells. Unintegrated DNA of SR-RSV-D-infected cells was extracted by Hirt fractionation (12) 3 days after infection at a multiplicity of 0.1 FFU per cell. The yield of infectious Hirt supernatant RSV DNA corresponded to approximately 1 × 10^4 FFU from 3 × 10^8 cells.

The infectious Hirt supernatant DNA was analyzed by electrophoresis in agarose gels with the use of linear and closed circular DNAs of plasmid pmDm691 (molecular weight, 6.2 × 10^6) as markers (Fig. 2). Infectious RSV DNA yielded a single peak in the position of linear DNA of approximately 6 × 10^6 daltons. No infectivity

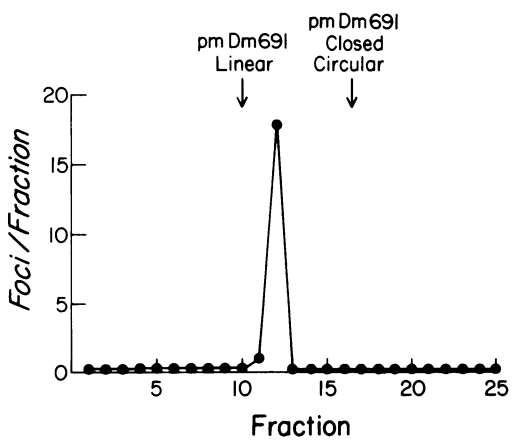

FIG. 2. *Agarose gel electrophoresis of Hirt supernatant RSV DNA. Hirt supernatant SR-RSV-D DNA was subjected to electrophoresis for 10 h at 30 V in a 0.7% agarose gel which contained 0.1 M NaCl and 4 μg of ethidium bromide per ml. The gel was fractionated into 3-mm slices, and the DNA was eluted and assayed for infectivity. Marker DNAs of plasmid pmDm691 (molecular weight, 6.2 × 10^6) were subjected to electrophoresis in a parallel gel.*

(<10%) was detected in the position of closed circular DNA. Therefore, the infectious Hirt supernatant DNA consisted primarily of unintegrated linear RSV DNA molecules.

The results of transfection assays of unintegrated SR-RSV-D DNA by both focus and colony formation are presented in Table 3. The Hirt supernatant DNA was infectious for C/O cells but not for C/BDE cells (resistant to infection by SR-RSV-D). Therefore, transfection by unintegrated linear RSV DNA also appeared to require formation of extracellular progeny virus and secondary virus infection.

Production of progeny virus by resistant cells exposed to RSV DNA. Since transfection appeared to proceed by transcription of donor DNA and formation of extracellular progeny virus, it was of interest to investigate virus production by resistant cells which had been exposed to RSV DNA.

C/ACE cells (resistant to infection by B77V-C) were exposed to B77V-C DNA, the DNA was removed, and cells which were sensitive to infection by subgroup C RSV (C/E or T/BD cells) were added to determine whether the DNA-treated C/ACE cells could serve as infectious centers. T/BD cells were resistant to transfection by B77V-C DNA (unpublished data) and were therefore used in this experiment to exclude the possibility that transfection could occur by uptake of residual DNA by the added sensitive cells. The specific infectivity of B77V-C DNA was approximately 6 FFU/μg on C/E cells, but less than 0.1 FFU/μg on C/ACE cells (Table 4). However, addition of C/E or T/BD cells to DNA-treated C/ACE cell cultures resulted in focus formation at frequencies of 2 to 3 FFU/μg of DNA (Table 4). Therefore, C/ACE cells served as infectious centers after exposure to B77V-C DNA with an efficiency comparable to the efficiency of transfection of sensitive cells.

The kinetics of production of progeny virus after exposure of sensitive or resistant cells to B77V-C and SR-RSV-D DNAs are presented in Fig. 3. Progeny virus was first detected 2 days after DNA treatment of either sensitive or resistant chicken embryo fibroblasts. The titer of progeny virus produced by sensitive cells increased to 10^7 to 10^8 FFU of B77V-C or to 10^6 FFU of SR-RSV-D per culture by 7 to 10 days after DNA treatment, at which time transformation of the DNA-treated cultures approached confluency. DNA-treated resistant cells produced progeny virus at a titer of 10^2 to 10^3 FFU per culture for at least 10 days after exposure to DNA, although no foci of transformed cells were evident.

The persistence of progeny virus production for 10 days after transfection of resistant cells suggested that stably transformed cells might be present at later times after DNA infection. To test this possibility, C/BDE cells (resistant to infection by SR-RSV-D) were exposed to SR-RSV-D DNA, maintained under liquid medium for 8 days, and then transferred into soft agar. Transformed cell colonies were obtained with a frequency corresponding to approximately 10 colony-forming units/μg of DNA, indicating that genetically stable transformation had occurred.

Stable transformation of resistant cells 8 days after transfection could be due either to direct integration of donor RSV DNA or to exogenous virus infection of the resistant cells during co-cultivation with cells which were producing progeny RSV via transcription of unintegrated donor RSV DNA. Crittenden (6) previously observed that C/A cells were susceptible to infection with subgroup A RSV during co-cultivation with lethally irradiated RSV-A-producing cells. To test this possibility in the present experiments, C/BDE cells were killed 2 days after transfection with SR-RSV-D DNA by treatment

TABLE 3. *Transfection by unintegrated SR-RSV-D DNA*[a]

DNA	Recipient cells	Foci/plate[b]	Colonies/plate[b]
SR-RSV-D Hirt supernatant	C/O	7	12
	C/BDE	0	0
B77V-C	C/O	24	50
	C/BDE	9	48

[a] Recipient cultures of C/O or C/BDE chicken embryo fibroblasts were exposed to SR-RSV-D Hirt supernatant DNA or to 5 μg of B77V-C DNA. Cultures were either maintained under liquid medium or transferred into soft agar 2 days after DNA treatment.

[b] Average of quadruplicate plates.

TABLE 4. *Infectious center assay of virus production by resistant cells exposed to RSV DNA*[a]

Phenotype of DNA-treated cells	Phenotype of cells added after removal of DNA	Foci/plate[b]
C/E	None[c]	33
C/ACE	None[c]	0
C/ACE	C/E	17
C/ACE	T/BD	9

[a] Cultures of C/E or C/ACE chicken embryo fibroblasts were incubated for 4 h with 5 μg of B77V-C DNA. The DNA was removed, and C/E or T/BD cells were added to some cultures. The cells were maintained under liquid medium and foci were counted 7 days after DNA treatment.

[b] Average of triplicate plates.

[c] No cells were added after DNA treatment of these cultures.

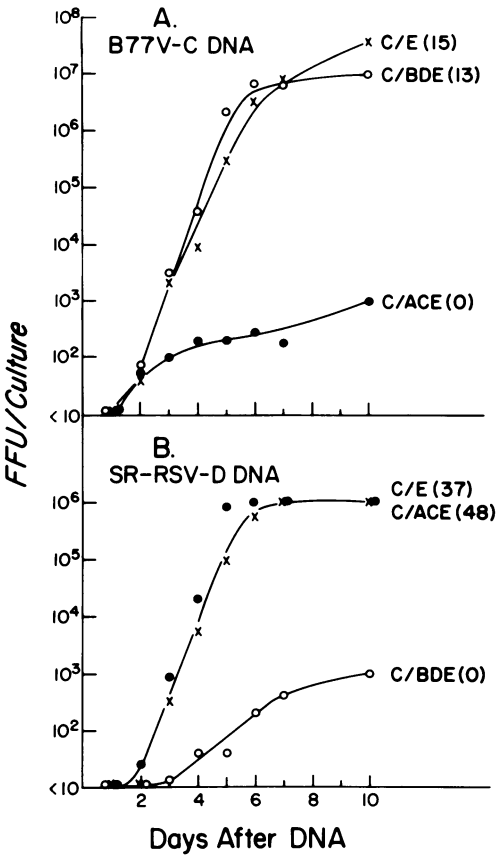

FIG. 3. *Kinetics of progeny virus formation. Duplicate cultures of C/E (×), C/ACE (●), or C/BDE (○) chicken embryo fibroblasts were exposed to 5 μg of B77V-C (panel A) or SR-RSV-D (panel B) DNAs. Supernatant media harvested at 24-h intervals were assayed on C/O cells to determine the titer of progeny virus (FFU/culture). The number of foci on the original DNA-treated recipient cell cultures, counted 6 days after exposure to DNA, are indicated in parentheses after each cell phenotype.*

with mitomycin C, co-cultivated with fresh C/BDE cells, and transferred into soft agar 8 days after exposure to DNA. Transformed cell colonies were obtained with a frequency corresponding to 5 to 10 colony-forming units/μg of DNA, indicating that exogenous virus infection of resistant cells did occur during co-cultivation with DNA-treated virus-producing cells. To test further the possibility that direct integration of donor RSV DNA might occur, C/ACE cells were exposed to BHT(RAV-1) DNA, maintained under liquid medium for 8 days, and transferred into soft agar. No transformed cell colonies were obtained (less than 0.01 colony-forming unit/μg of DNA). Therefore, it appeared that genetically stable transformation of resistant cells 8 days after exposure to DNA was due to exogenous virus infection rather than to donor DNA integration.

DISCUSSION

In this paper we report genetic experiments in which we investigated the mechanism of transfection of chicken cells by RSV DNA. Recipient chicken cells which were resistant to virus infection as a result of the absence of receptors required for virus penetration were also resistant to transformation by RSV DNA. In addition, DNA of defective BH-RSV (*env*$^-$) was noninfectious, and transfection by DNA of *LA335* (*pol*^{ts}) was temperature sensitive. These results indicated that secondary virus infection was necessary for transformation by RSV DNA. Since transformation was assayed by colony formation in soft agar, as well as by focus formation, the requirement for secondary virus infection was not an artifact of possible difficulties in detection of foci formed only by cell division. Thus, exposure to RSV DNA did not result in genetically stable transformation of the primary DNA-treated recipient cells. We have considered three hypotheses to account for these results.

(i) Transfection proceeds by integration of donor RSV DNA, but results in killing of the primary DNA-treated recipient cells. However, we observed that cells which were resistant to secondary virus infection produced progeny virus, indicating that the primary DNA-treated recipient cells remained viable. Nevertheless, we cannot exclude the possibility that these cells, although metabolically active and able to produce virus, were unable to divide to form foci or colonies in soft agar.

(ii) Donor RSV DNA is integrated in primary DNA-treated recipient cells, but the transformation gene (*src*) is not expressed. Since resistant cells transfected by RSV DNA were found to produce progeny virus, this hypothesis assumes a specific lack of expression of the *src* gene. This appears unlikely, particularly if *env* and *src* mRNA's are derived from 35S RNA by a similar splicing mechanism (19, 30).

(iii) Transfection proceeds by transcription of unintegrated donor DNA, formation of extracellular progeny virus, and secondary virus infection. Expression of the *src* gene of donor RSV DNA could result in phenotypic transformation of primary DNA-treated recipient cells, but transformation would be abortive if the unintegrated donor DNA were not stably transmitted to daughter cells.

Since genetically stable transformation required formation of extracellular progeny virus, it appeared that synthesis and integration of

progeny RSV DNA did not occur in the same cell which was the primary recipient for uptake and transcription of donor RSV DNA. Formation of extracellular virions might be required for completion of processes which occur during virion assembly or maturation. Such processes could include formation of the 70S RNA complex (1, 2), formation of DNA polymerase by precursor polypeptide cleavage (20), or association of DNA polymerase with primer and template RNAs.

In contrast to the present observations, transformation of some mouse cell lines by DNA of defective murine sarcoma virus occurs at high efficiency, indicating that murine sarcoma virus DNA integrates directly into the recipient mouse cell genome (14, 16, M. Goldfarb and R. A. Weinberg, personal communication). We have confirmed these results with murine sarcoma virus DNA and have also demonstrated transformation of NIH 3T3 mouse cells by SR-RSV-D DNA (N. Copeland and G. M. Cooper, unpublished data). Thus, it appears that transfecting retrovirus DNAs integrate efficiently in NIH 3T3 mouse cells, but not in chicken embryo fibroblasts. The basis of this difference in transfection might be related to the recipient cell species, to the fact that NIH 3T3 cells are an established cell line, or to factors which might be unique to the particular cells studied. The different fates of donor DNAs in different recipient cells might be related to differences in the cellular contents of enzymes involved in DNA integration.

The lack of direct transformation of chicken cells by RSV DNA also differs from the transforming activity of fragments of papova virus, adenovirus, and herpes simplex virus DNAs (reviewed in 9). However, the efficiency of transformation by fragments of these viral DNAs is generally less than 1 FFU per 10^8 viral genome equivalents (9). Since this is approximately 10^3-fold lower than the efficiency of transfection by RSV DNA, we would not have detected a similar frequency of direct transformation by RSV DNA in the present experiments.

Transfection by unintegrated linear RSV DNA, as well as by total cell DNA, appeared to proceed by transcription of the donor DNA. However, unintegrated linear DNA synthesized in the cytoplasm of RSV-infected cells appears to be the precursor of closed circular DNA (23), which is the probable substrate for integration. Therefore, the activity of unintegrated virus DNAs in transfection assays (7, 11, 26, 27) may not be indicative of their possible roles as precursors of integrated proviral DNA in virus-infected cells. If transfection proceeds by transcription of unintegrated donor DNA, the infectivity of unintegrated linear RSV DNA also indicates that transcription of viral DNA can occur in the absence of adjacent cellular DNA sequences. Since significant amounts of unintegrated viral DNAs may persist for several weeks after infection (7, 11, 28), these observations suggest the possibility that these unintegrated DNAs, as well as integrated proviral DNA, might serve as templates for viral RNA synthesis.

The hypothesis that transfection by RSV DNA proceeds via transcription of donor DNA is also relevant to the interpretation of experiments in which transfection was used to study the endogenous RAV-0 DNA of uninfected chicken cells (5). In contrast to the infectivity of RAV-0 proviral DNA of RAV-0-infected cells, the endogenous RAV-0 genome of uninfected V^+ chicken cells was not infectious (5). Since the titer of RAV-0 produced by uninfected V^+ cells was 10^3- to 10^4-fold lower than the titer of RAV-0 produced by RAV-0-infected cells (5, 25), it was proposed that the lack of infectivity of the endogenous RAV-0 genome was a consequence of linkage to a *cis*-acting control element which inhibited its transcription (5). The present results indicate that transcription of donor DNA is a primary event in transfection and thereby clarify a possible mechanism by which a linked *cis*-acting transcriptional control element could affect the infectivity of endogenous RAV-0 DNA.

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