Replication of Endogenous Avian Retrovirus in Permissive and Nonpermissive Chicken Embryo Fibroblasts

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Clones of chicken embryo fibroblasts exogenously infected with the endogenous avian retrovirus were analyzed to examine the replication of this virus in permissive (Gr^+) and nonpermissive (Gr^-) cells. The results demonstrate that the endogenous virus was capable of infecting both Gr^+ and Gr^- cells with equal efficiency. Infected clones of Gr^+ and Gr^- cells differed, however, in two significant ways. At the time of their initial characterization, the Gr^+ clones produced 100- to 1,000-fold more virus than the Gr^- clones. Further, the amount of virus produced by Gr^+ clones did not change significantly during serial passage of the cells. In contrast, continued passage of the infected Gr^- clones resulted in a gradual increase in the amount of virus produced. Individual clones of infected Gr^- cells produced infectious virus at rates that, initially, differed by a factor of more than 10^4 . The large differences in the production of virus by these clones could not be explained by equally large differences in the number of infected cells within the clonal populations. Greater than 80% of the clonal populations examined ultimately produced virus at rates that were not significantly different from the rates observed in infected Gr^+ cells. Virus produced by these infected Gr^- cells exhibited the same restricted replication upon establishing a new infection in nonpermissive cells. Analysis of the appearance of free and integrated viral DNA sequences during endogenous virus infection of Gr^+ and Gr^- cells demonstrated that, after an initial delay in the synthesis of free viral DNA in Gr^- cells, the nonpermissive cells ultimately acquired as many integrated viral DNA sequences as were found in infected Gr^+ cells. These results indicate that a majority of the infectious particles of the endogenous virus are capable of establishing infection in a Gr^- cell and, ultimately, of producing virus at a rate that is not significantly different from that produced by infected Gr^+ cells. The virus produced from the Gr^- cells is not a stable genetic variant of the original endogenous virus that is capable of unrestricted replication in nonpermissive cells. The reduced efficiency with which the endogenous virus initially replicates in nonpermissive cells and the increased length of time required for infected Gr^- cells to produce maximal virus titers suggest that the endogenous virus may utilize a different mechanism of replication in Gr^+ and Gr^- fibroblasts.

Chickens contain a number of genetic loci that are related to the viruses of the avian leukosis-sarcoma virus complex (1, 22, 26). Some of these loci code for infectious virus. These viruses, of which Rous-associated virus 0 (RAV-0) is the prototype, are classified as endogenous avian retroviruses (2, 5, 6, 27, 30). Since these loci are normally expressed at low levels (4, 14, 22, 28, 31), endogenous virus can be isolated from cultured embryonic fibroblasts derived from several inbred lines of chickens. The replication of the endogenous virus in chicken cells is influenced by at least three host functions. First, a majority of chickens do not possess the receptor specific for the subgroup E envelope glycoprotein of the virus. Therefore, exposing fibroblasts from these chickens to the endogenous virus does not result in viral attachment and penetration (7, 28, 30). Second, chickens that do express the appropriate receptor frequently coexpress a glycoprotein which binds specifically to the receptor, thus preventing attachment of the endogenous virus (7, 10, 20, 23, 32). Third, an intracellular restriction that inhibits the replication of the subgroup E endogenous viruses in cultured chicken fibroblasts has been described (19, 21, 30). This restriction, which results in a decreased rate of endogenous virus replication, is expressed in all fibroblasts that have been examined except those prepared

from RPRL (Regional Poultry Research Laboratory) line 15_B chickens. At present, only fibroblasts from these chickens are considered fully permissive for endogenous virus replication. These permissive cells have been assigned the Gr^+ phenotype, whereas nonpermissive cells are designated Gr^- . The Gr^+ phenotype is expressed by a dominant allele (21).

The restriction is characterized by a marked reduction in the rate at which the endogenous virus replicates in newly infected cultures of Gr^- cells. This restriction is specific for endogenous viruses since exogenous avian retroviruses replicate equally well in newly infected cells of both types. Previous work has suggested that continued culture of the Gr^- cells infected with endogenous virus results in an increase in the rate of virus production to a level that is reduced only fivefold from that produced by infected Gr^+ cells (21). However, since RAV-60, a recombinant exogenous virus that has a subgroup E envelope glycoprotein (9), is also produced from Gr^- cells at slightly reduced levels, it is not clear that this difference is related to the restriction that inhibits the initial replication of the endogenous virus.

Analysis of the replication of the endogenous virus in nonpermissive cells has shown that after infection there is 10-fold less viral DNA synthesis than is detected in infected permissive cells (12). Whether the intracellular restriction that inhibits endogenous virus replication is related to the reduced rate of DNA synthesis is unknown. The reduced

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rate with which the endogenous virus replicates in Gr⁻ cells, in conjunction with the reduction in viral DNA synthesis observed in Gr⁻ cells, has prompted the suggestion that the restriction in Gr⁻ cells may involve an early replication event such as attachment or penetration of the subgroup E virus. It has been reported that variants of the endogenous virus capable of replicating with an increased efficiency in the nonpermissive cell can be isolated after long-term virus infection of these cells (21). Analysis of the synthesis of viral DNA in nonpermissive cells infected by these variants, however, does not show an increased rate of synthesis when compared with the DNA synthesis exhibited by the parent endogenous virus (12).

The present study was initiated to determine what proportion of the infectious particles of the endogenous virus were capable of initiating replication in nonpermissive cells and, further, what proportion of those infectious particles were able to give rise to variants capable of unrestricted growth in Gr⁻ cells. We examined endogenous virus infection and replication in permissive and nonpermissive cells by analyzing clonal cell populations prepared from cells that had been exposed to virus. Although we were unable to detect stable variants of the endogenous virus, our results demonstrate that a majority of the virus particles are capable of infecting and replicating in the nonpermissive cells. (For the purposes of this paper, the establishment of infection of a cell by the endogenous virus will be defined as the production of infectious virus by that cell. This does not necessarily include the formation of an integrated DNA copy of the viral genome.) Although clones of virus-infected nonpermissive cells frequently produce very limited amounts of virus early after isolation of the clone, continued passage of these cells demonstrates that they ultimately produce virus at a rate that is not significantly different from that established in the permissive cell. The results of this study indicate that the endogenous virus infects both permissive and nonpermissive cells with equal efficiency. The rate at which the viral infection spreads in Gr⁻ cells, however, is significantly different from that observed in Gr⁺ cells. These results are consistent with the hypothesis that the endogenous virus replicates in nonpermissive cells by a mechanism that is distinct from that employed in permissive cells, and further, that this replication phenotype is a genetically stable property of the endogenous virus.

MATERIALS AND METHODS

Cells and viruses. All cells were cultured in plastic dishes (Nunc, Denmark) with Dulbecco modified Eagle medium (Flow Laboratories, Bethesda, Md.) containing 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) supplemented with calf serum and fetal calf serum (Sterile Systems, Logan, Utah) in a humidified atmosphere at 38°C containing 5 to 10% CO₂.

RPRL line 15_B fibroblasts used in this study were prepared from a single embryo and contained the endogenous viral loci 1 and 7 (*ev-1* and *ev-7*). They are C/C, chicken-helper factor negative (*chf*⁻), and permissive for endogenous virus replication and therefore designated Gr⁺ as described previously (13). Fibroblasts were also prepared from a single chicken embryo of the SC line (Hyline International, Dallas Center, Iowa). This embryo contains the endogenous viral loci *ev-1* and *ev-4* (E. Humphries, M. Danhof, and I. Hlozaneck, Virology, in press). These cells are C/0, *chf*⁻, V⁻, and Gr⁻. The Reaseheath line C fibroblasts have been described previously (13). They contain *ev-1*, *ev-7*, and *ev-10*. They are C/E, *chf*⁻, and V⁺. Chicken embryo fibroblasts

(CEF) from a cross designated [15_B × K(-)] × K₂₈ were obtained from H. Robinson (Worcester Foundation for Experimental Biology, Shrewsbury, Mass.) (21). These cells are C/0, *chf*⁻, V⁻ and Gr⁺. K₂₈ CEF were also obtained from H. Robinson (25). These cells are C/0 and Gr⁻.

Reaseheath line C fibroblasts spontaneously produce an endogenous virus derived from *ev-10* (5) that is designated EV-C. Cultures of Reaseheath line C fibroblasts produce titers of this virus that range from 10 to 100 IU/ml. This virus has a subgroup E glycoprotein and exhibits restricted replication in Gr⁻ chicken cells (21). RAV-60 was obtained from H. Robinson (21).

Cloning of CEF. CEF were cloned by the procedure of Beug and Graf as described earlier (3, 13). The cells used for cloning were usually at the second or third passage after preparation of primary cultures. The cloning efficiency in our experiments, as defined by the number of clones that reach 10⁷ cells, was ca. 5%. Two methods were used to prepare EV-C-infected cell clones. Line 15_B or SC CEF were plated at 10⁶ cells per 50-mm plate. Four hours after plating, the cells were exposed to EV-C with a multiplicity of infection of ca. 1. Four hours after infection, the cells were cloned either by plating 100 cells per 90-mm plate or 1 cell per well in 96-well microtiter trays. The cells were then incubated at 38°C for 9 to 10 days. Individual colonies were transferred to 17-mm wells then to 35-mm plates then to 50-mm plates, at which point they were referred to as T₃ cultures. Two types of experimental protocols were employed to analyze virus production in the isolated clonal populations. In some experiments, the clones were maintained as clonal populations by serial transfer. The clones were cultured in 50-mm plates until they reached a density of ca. 4 × 10⁶ or 5 × 10⁶ cells per plate, at which time 1/10 of the cells were transferred to a new plate. Clones analyzed in this fashion could usually be transferred three or four times (T₆ or T₇ cultures). In other experiments, the isolated clones were maintained and analyzed as clonal populations only as far as T₄ cultures. T₅ cultures were established by plating 5 × 10⁵ cells from the clone with 5 × 10⁵ cells from the uncloned parental SC embryo. These cultures were maintained for an additional six to seven transfers, at which point they were referred to as T₁₁ and T₁₂ cultures. Analysis of these cells represents, therefore, from the T₅ culture onward, the replication of a clonal population of viruses in a nonclonal population of cells.

Virus titrations and infectious center assays. Two methods were used routinely to detect virus production. Overnight growth medium was harvested from a 50-mm plate, clarified by low-speed centrifugation, and concentrated by centrifugation at 30,000 rpm for 1 h at 4°C in a Beckman type 40 rotor. Virus pellets were resuspended in 50 μl of 50 mM Tris-hydrochloride (pH 8.0)–5 mM EDTA. The concentrated samples were stored at -20°C until they were assayed. The details of the RNA-directed DNA polymerase assay have been described previously (13). The results of the assay are presented as the number of counts per minute incorporated for a 40-min reaction by the virus produced from ca. 3 × 10⁶ to 5 × 10⁶ cells during a 16-h interval. Production of virus was also determined by assay for infectious particles. All viral samples were collected in 0.5 ml of growth medium per 50-mm plate and harvested after 2 h at 38°C. The samples were assayed by infecting 35-mm plates containing [15_B × K(-)] × K₂₈ (Gr⁺) cells with 50 μl of different dilutions. The Gr⁺ cells were cultured until confluent and transferred to 50-mm plates at 6 × 10⁵ cells. These cells were cultured and transferred once, and the supernatants from 50-mm plates

containing ca. 3×10^6 to 5×10^6 cells were harvested and assayed for the presence of RNA-directed DNA polymerase activity. The titer of these virus preparations was determined by the standard 50% tissue culture infective dose procedure. Titration of stock virus preparations was carried out by infecting five plates per dilution. Titrations of samples produced from cloned cell populations were carried out by infecting two plates per dilution.

Cells were assayed by an infectious center procedure by plating different concentrations of cells in 35-mm plates containing 2.5×10^5 Gr⁺ cells. These cells were cultured for 2 or 3 days and transferred to 50-mm plates at 6×10^5 cells per plate. These cells were cultured, transferred once, and cultured until there were ca. 4×10^6 cells per plate. The growth medium was then harvested and assayed for the presence of RNA-directed DNA polymerase activity as described above. The standard assay plated 0.1, 1, 10, and 100 cells per 35-mm plate, with five plates per dilution. The percentage of cells positive for virus production was determined from the number of plates from which DNA polymerase activity was detected at each dilution.

Preparation of cellular DNA. Cellular DNA was prepared as described previously (13). Briefly, the cells were lysed with 0.5% sodium dodecyl sulfate and 100 μ g of proteinase K per ml. After digestion at 38°C, the DNA was extracted with phenol and chloroform. After dialysis against 10 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl–10 mM EDTA, the DNA preparation was digested with RNase A, digested with proteinase K, and extracted with phenol and chloroform. After dialysis against 10 mM Tris-hydrochloride (pH 8.0)–10 mM NaCl–1 mM EDTA, the DNA was analyzed spectrophotometrically for purity and concentration. DNA samples were not precipitated since detection of low-molecular-weight DNA species was required.

Restriction enzyme analysis, Southern DNA transfer, and nucleic acid hybridization. The methods used have been described previously (13, 14). *SacI* and *HhaI* restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.). The DNA probe was an equimolar mixture of two subclones of RAV-0 provided by S. Hughes (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). They contained ca. 95% of the viral genome. These subclones, R8-HR-13 and R8-HR-16, were derived from the R8 clone by *HindIII* and *EcoRI* codigestion (11). They were labeled by nick translation to a specific activity of ca. 2×10^8 cpm/ μ g of DNA.

RESULTS

Endogenous virus infection of permissive and nonpermissive chicken fibroblasts. The purpose of the present work was twofold. First, we wanted to evaluate the hypothesis that nonpermissive cells inhibited the replication of the endogenous virus by reducing the efficiency of virus infection. Such a restriction might be the consequence of a reduced rate of attachment and penetration mediated by the subgroup E glycoprotein (12, 21). We wanted, therefore, to determine the efficiency with which the endogenous virus could infect permissive (Gr⁺) and nonpermissive (Gr⁻) cells. Second, we wanted to determine what proportion of the infectious events mediated by the endogenous virus could give rise to a variant capable of nonrestricted growth in Gr⁻ cells (21). For these experiments we chose to use $[15_B \times K(-)] \times K_{28}$ fibroblasts as the permissive (Gr⁺) cells. These cells do not appear to release endogenous virus or endogenous virus recombinants spontaneously (24). A few early experiments used cells from an embryo of line 15_B that has been in culture

FIG. 1. Cocultivation of Reaseheath C line CEF with permissive (Gr⁺) and nonpermissive (Gr⁻) cells. In 50-mm plates 4×10^5 Reaseheath C (RC) CEF and either 4×10^5 $[15_B \times K(-)] \times K_{28}$ (Gr⁺) CEF or 4×10^5 SC (Gr⁻) CEF were cultured in growth medium. At 2- to 3-day intervals, the growth medium was harvested to determine the amount of DNA polymerase activity present, and 1/10 of the cells were transferred to new plates.

for several years and has never produced spontaneous endogenous virus. The fibroblasts from the Hyline SC chicken were used as nonpermissive cells (Gr⁻) because they can be cloned relatively easily. The endogenous virus produced from the Reaseheath C line fibroblasts was used throughout this study. This virus, designated EV-C, is produced from *ev-10* at very low efficiency (21). EV-C was chosen for this study because the viral DNA has no cleavage site for *SacI* endonuclease (E. H. Humphries, unpublished data).

To characterize the replication of EV-C on permissive and nonpermissive cells, Reaseheath line C (CEF) were cocultivated with $[15_B \times K(-)] \times K_{28}$ CEF or SC CEF. The cells were cultured in growth medium and transferred at 2- to 3-day intervals. The amount of virus present in the cultures was determined by assaying the DNA polymerase activity present in the growth medium before cell transfer. The results (Fig. 1) demonstrate that DNA polymerase activity was first detected at the third transfer of the cocultivation with Gr⁺ cells and at the fifth transfer of the cocultivation

TABLE 1. Endogenous virus infection of permissive (Gr⁺) and nonpermissive (Gr⁻) CEF^a

Expt.	Gr ⁺ CEF Clones					Gr ⁻ CEF Clones				
	Polymerase activity produced (no.) ^b			Infectious virus produced (no.) ^c		Polymerase activity produced (no.)			Infectious virus produced (no.)	
	+	+/-	-	+	-	+	+/-	-	+	-
1	3	0	8	ND ^{d,e}	ND	0	0	16	5 ^f	5
2	18	3	2	21	2	4	3	41	45	3

^a Line 15_B and SC CEF were exposed to EV-C at a multiplicity of ca. 1.0. Four hours later, the cells were cloned as described in the text. In both experiments, the clones were maintained as clonal populations throughout the analysis.

^b Growth medium was harvested at T₃ and T₄ (experiment 1) and T₃, T₅, and T₇ (experiment 2) and assayed for DNA polymerase activity as described in the text. Values ≤ 400 cpm for incorporation of [³H]TMP in a 40-min reaction were considered negative (-). Values between 400 and 1,500 cpm were considered +/- . Growth medium from uninfected cells incorporated ca. 200 cpm.

^c Growth medium was harvested at T₅ (experiment 1) and T₃ (experiment 2) and assayed on Gr⁺ cells for EV-C.

^d ND, Not determined.

^e Since the number of Gr⁺ clones producing infectious virus was not determined, it is possible that the number of infected Gr⁺ clones was greater than the 3 of 11 positive for DNA polymerase activity. Although infected line 15_B clones that are negative for DNA polymerase activity and still release infectious virus may exist (13), they will be relatively infrequent.

^f Only 10 clones were analyzed for virus production.

with Gr⁻ cells. Continued growth and transfer of the Gr⁻ cell cocultivation ultimately produced a level of DNA polymerase activity that was not significantly different from that detected in the Gr⁺ cocultivation (data not shown). This experiment was typical of (and qualitatively similar to) several that have been performed by using either cocultivation with Reaseheath line C CEF or direct infection of Gr⁺ and Gr⁻ CEF with filtered growth medium harvested from the Reaseheath line C cells. This experiment, therefore, defined the nonpermissive phenotype of the SC fibroblast as a delay in the appearance of DNA polymerase activity that was indicative of a reduction in the initial rate of endogenous virus replication in this cell. The extent of the delay in the replication of EV-C in the SC fibroblasts was comparable to the delay in viral replication observed in the K₂₈ fibroblast (21; data not shown).

To study the nonpermissive nature of the SC fibroblasts, experiments were conducted to analyze the efficiency with which endogenous virus could infect the Gr⁻ cells. Preliminary experiments in the form of endpoint dilution titrations on both Gr⁺ and Gr⁻ fibroblasts indicated that EV-C could infect both cell types with approximately equal efficiency. To study this point further, both line 15_B and SC fibroblasts were infected with EV-C at a multiplicity of ca. 1. Four hours after infection, the cells were trypsinized and replated at a density of ca. 100 cells per 90-mm plate in cloning medium. The clones were maintained as clonal populations throughout the experiment. At different times, growth medium was harvested and assayed for the presence of DNA polymerase activity or infectious virus. The results from two separate experiments indicate that the endogenous virus infected both Gr⁺ and Gr⁻ CEF with equal efficiency (Table 1). Of the 21 clones of Gr⁺ CEF that produced infectious virus, all produced detectable DNA polymerase activity. In contrast, of the 50 clones of Gr⁻ cells that produced infectious virus, only 7 produced detectable polymerase activity. These data are consistent with the observation that EV-C replicated poorly in Gr⁻ cells for three or four culture transfers (Fig. 1). It should be noted that all of the Gr⁻ clones that produced detectable DNA polymerase activity were observed in experiment 2 and examined at T₃, T₅, and T₇ cultures. In contrast, the clones in experiment 1 were examined for DNA polymerase activity in T₃ and T₄ cultures. These data suggested, therefore, that clones of Gr⁻ cells infected with the endogenous virus were more likely to

produce detectable polymerase activity the longer they were kept in culture.

The clones isolated in experiments 1 and 2 were isolated from 90-mm plates that had been seeded with ca. 100 cells. As a control to ensure that each of the clones isolated was infected at the time of exposure to the original virus inoculum and not infected in the cloning medium by virus produced from one particular clone, individual clones were prepared by seeding trays of microwells with one cell per well. Of 23 clones prepared after infection of Gr⁻ cells at a multiplicity of ca. 1 virus per cell, 9 produced infectious virus when assayed at the T₃ culture. The results presented in Fig. 1 and Table 1 demonstrate that the endogenous virus was capable of infecting SC fibroblasts with an efficiency equal to that observed during infection of the permissive line 15_B fibroblast. Therefore, although the SC fibroblast may be considered nonpermissive in that the initial rate of viral replication was reduced, a majority of the cells permitted attachment, penetration, and replication of the endogenous virus, demonstrating that the rate of replication of the endogenous virus is not restricted by a surface-mediated mechanism.

Replication of clonal populations of EV-C in Gr⁻ cells. Although 45 of 48 clones produced infectious virus, most clones produced too little virus to be detected in the DNA polymerase assay (Table 1, experiment 2). Further, of the seven clones which produced detectable DNA polymerase activity in the T₇ culture, six were negative in the DNA polymerase assay in the T₃ culture (data not shown). To examine the possibility that a majority of the infectious EV-C particles could infect Gr⁻ cells and produce sufficient virus to be detected in the DNA polymerase assay, the experiment was repeated by using long-term cultures of Gr⁻ cells infected with different clones of the endogenous virus. Gr⁻ CEF were infected with EV-C at a multiplicity of ca. 1 and cloned by plating 100 cells in a 90-mm plate in cloning medium. Twenty-seven clones were picked and carried as clonal populations to the T₄ culture (Table 2). These clones were examined for the production of infectious virus and DNA polymerase activity in the T₃ culture. The T₅ culture of all 27 clones was established by plating an equal number of cells from each clone with uninfected, uncloned Gr⁻ cells of the parental embryo. In this way, each clonal population of virus, present in a cellular clone, could be maintained in the Gr⁻ cells for an additional six transfers. All clones were

TABLE 2. Replication of clonally derived populations of EV-C in nonpermissive CEF^a

Clone	Production of DNA polymerase activity ^b					Production of infectious virus ^c		
	T ₃	T ₅	T ₇	T ₉	T ₁₁	T ₃	T ₇	T ₁₁
1	+/-	+	+	+	+	+	ND ^d	+
2	-	-	+	+	+	+	+	+
3	-	-	+/-	+	+	+	ND	+
4	-	-	-	+/-	+	+	ND	+
5	-	-	-	+	+	+	ND	+
6	-	-	-	+	+/-	+	ND	+
7	-	-	-	-	+	+	ND	+
8	+	-	-	+	+	+	ND	+
9	+	-	-	+	+	+	+	+
10	-	-	-	+/-	-	+	-	+
11	-	-	-	-	+/-	+/-	-	+/-
12	-	-	-	-	-	-	+	+
13	-	-	-	-	-	-	+	+/-
14-27	-	-	-	-	-	-	-	-

^a Initial infection and cloning of Gr⁻ cells was carried out as described in Table 1, footnote *a*. The T₅ culture of these clones was established by plating 4×10^5 T₄ cells with 4×10^5 uncloned Gr⁻ cells in a 50-mm plate. Subsequent transfers were established by plating 1/10 of the cells in a 50-mm plate as described in the text.

^b Growth medium from T₃, T₅, T₇, T₉, and T₁₁ cultures was assayed for DNA polymerase activity. The results of the assay were designated +, +/-, or - as described in Table 1, footnote *b*.

^c Growth medium from T₃, T₇, and T₁₁ cultures was assayed for the presence of infectious virus.

^d ND, Not determined.

examined for DNA polymerase activity and production of infectious virus (Table 2). Fourteen of the clones produced no infectious virus or DNA polymerase activity and were considered uninfected (clones 14 to 27). The remaining 13 clones all produced infectious virus. Although only one clone produced detectable DNA polymerase activity in the T₅ culture, 11 of the 13 clones produced detectable DNA polymerase activity by the T₁₁ culture (Table 2). The data in Table 2 demonstrate that continued propagation of Gr⁻ cells infected with clonally derived populations of EV-C produced cultures of infected Gr⁻ cells which released detectable DNA polymerase activity.

Analysis of the amount of pelletable DNA polymerase activity produced from the clones of Gr⁺ and Gr⁻ CEF infected with EV-C (Tables 1 and 2) revealed two additional points. First, the amount of virus produced from EV-C-infected Gr⁺ clones did not change significantly with passage of clonal populations (Fig. 2). Within the error of the assay (approximately two- to threefold), the majority of the line 15_B clones produced a constant amount of virus as noted earlier (13). The six clones chosen represent a random sampling of the Gr⁺ clones isolated during this experiment. Consistent with previously published data, the amount of virus produced by different Gr⁺ clones varied ca. 20-fold (13, 15). Second, Gr⁻ cells infected with clonal populations of EV-C produced increasing amounts of DNA polymerase activity with continued culture transfer (Fig. 3). The amount of DNA polymerase activity detected in clones that were transferred 11 times was not significantly different from that detected in cultures of Gr⁺ cells.

The data presented (Fig. 2 and 3 and Table 2) demonstrate that a majority of the infectious particles of EV-C could establish infection in Gr⁻ cells. After prolonged culture, these infections produced an amount of DNA polymerase activity equal to that produced by EV-C-infected Gr⁺ cells. It appears, therefore, that the pattern of replication of the endogenous virus in a nonclonal Gr⁻ population (Fig. 1) was representative of a majority of the infection events examined clonally.

Analysis of Gr⁻ CEF infected with EV-C. To study further

the replication of the endogenous virus in clonal populations of Gr⁻ cells, nine clones of Gr⁻ CEF were analyzed for production of infectious virus and for infectivity in infectious center assays. The clones were examined as T₃ cultures. The amount of virus produced from these cultures during 2 h of incubation differed by 10⁵-fold (from less than 10 IU/ml to 10⁶ IU/ml; Table 3). Two clones that produced 10⁶ IU/ml (clones 1 and 8), were also positive for production of DNA polymerase activity (Table 2). Clones producing 10⁴ IU/ml or less were negative for production of DNA polymerase activity. Six of the nine clones were assayed for activity in the infectious center assay. There was a difference of ca. 40-fold (from 2 to 80%) in the ability of the cells from these six different clones to function as infectious centers. The same six clones examined in the infectious center assay were also examined for the presence of infectious virus in culture fluid. The amount of infectious virus produced from these clones differed by a factor of 10⁴. The data from the analysis of clones 3 and 8 are illustrative of a general observation. Although infectious center assays established that ca. 80% of the cells in both clones were infected, these clones exhibited a 100-fold difference in the amount of virus they were producing. Other examples that illustrate this phenomenon are presented in Table 3. Therefore, the difference in the rate at which virus was produced from these clones could not be explained by an equally large difference in the number of infected cells in these clones. For comparison, two clones of Gr⁺ cells infected with EV-C were analyzed in an identical manner. Both clones were producing greater than 10⁶ IU/ml. Cells from these clones scored as 60 and 80% positive in infectious center assays.

In five of the six clones examined, 20% or more of these cells were producing virus. The data suggest that as a clone expands from a single infected cell, a large proportion of the daughter cells continue to be infected. It should be pointed out, however, that only a small percentage of the cells in clone 4 were infected, despite the fact that it was isolated in parallel with the other eight clones. Because only a small sampling of clones was subjected to analysis by the infectious center assay, the proportion of such clones among

FIG. 2. Production of DNA polymerase activity from clones of Gr⁺ and Gr⁻ CEF infected with EV-C. The data are from the analysis of the clones described in experiment 2 of Table 1. The DNA polymerase activity was determined in T₃, T₅, and T₇ cultures.

those isolated remains undetermined. It should also be noted that the extent to which the variation in virus replication in different clones is a result of heterogeneity in the original fibroblast population is unknown.

Absence of genetically stable variant viruses capable of nonrestricted replication in Gr⁻ fibroblasts. As stated earlier, the purpose of the present work was, in part, to determine with what efficiency the infectious particles of the endogenous virus gave rise to genetically stable variants capable of nonrestricted replication in Gr⁻ cells. Having determined that the majority of the particles of the endogenous virus could replicate in Gr⁻ cells and establish clonal populations in which a majority of the cells were producing virus, two different experimental approaches were used to identify the replication of variants. Stocks of endogenous virus likely to contain variants were prepared as follows. SC cells were infected with EV-C at a multiplicity of ca. 0.001. The infected cells were cultured for 10 to 14 days with five to seven serial transfers. From three separate infections, stocks of the endogenous virus containing 10⁶ IU/ml or greater were harvested from the Gr⁻ cells. These viruses were designated EV-C.*

The replication of EV-C* was examined first by analyzing the infection of cultures of Gr⁺ and Gr⁻ CEF. Cultures of Gr⁺ and Gr⁻ cells were infected with EV-C* at multiplicities of 0.00001 and 0.01. For comparison, Gr⁺ and Gr⁻ cells were also infected with EV-C at the same multiplicities. These cells were cultured in growth medium and transferred at 2 to 3-day intervals. Before each transfer, the growth medium was harvested and assayed for the presence of DNA polymerase activity. The results demonstrate that the rate at which EV-C* replicated was indistinguishable from the rate at which EV-C replicated (Fig. 4). In both experiments, replication of the two viruses proceeded significantly faster on Gr⁺ cells, producing growth curves indistinguishable from that observed during the original isolation of the endogenous virus (Fig. 1). Because it was suggested earlier (12) that a low multiplicity of infection facilitated the isolation of replication variants, this experiment was repeated with two preparations of EV-C* derived independently, using multiplicities of infection of 0.0001 and 0.00001. Identical results were obtained in all experiments. No evidence for an increased rate of viral replication was observed after infection with EV-C*.

A second approach was used to identify variant virus. Gr⁻

FIG. 3. Production of DNA polymerase activity from EV-C-infected Gr⁻ clones. The data are from the analysis of the clones described in Table 2.

CEF were infected with EV-C* at a multiplicity of ca. 1. Four hours after infection, these cells were trypsinized and plated at ca. 100 cells per 90-mm dish in cloning medium. The clones were isolated and characterized as described earlier for the experiment presented in Table 2. As a control, cell cloning was performed in parallel by infecting Gr⁻ CEF with EV-C. A total of 24 clones derived from Gr⁻ cells infected with EV-C* and 27 clones derived from Gr⁻ cells infected with EV-C were analyzed for the production of DNA polymerase activity (Table 4). It was expected that if the stock of EV-C* contained a variant virus capable of nonrestricted replication on Gr⁻ cells, clones producing DNA polymerase in T₃ and T₇ cultures ought to be isolated more frequently than after infection of Gr⁻ cells by EV-C. Five distinct phenotypes were observed in this experiment. The distribution of clones among these phenotypes demonstrates that the replication of EV-C* and EV-C proceeded at virtually the same rate. The distribution of clones in this experiment was similar to that observed in the experiment described in Table 2. These data indicate that replication of EV-C in the nonpermissive cell did not select for a stable variant capable of nonrestricted replication.

Analysis of free and integrated viral DNA in EV-C-infected Gr⁻ cultures. It has been reported that viral DNA synthesis in EV-C-infected Gr⁻ cells is reduced 10-fold compared with viral DNA synthesis in Gr⁺ cells (12). Integration of endogenous viral DNA in Gr⁻ cells has not been reported. It has been suggested that replication of the endogenous virus is inhibited at an early step in the nonpermissive cell (12). Since Gr⁺ and Gr⁻ cells seem equally susceptible to infection, we decided to examine the synthesis and integration of endogenous viral DNA in the nonpermissive SC fibroblast. SC or [15_B × K(-)] × K₂₈ fibroblasts were seeded at 1.5 × 10⁶ cells per 90-mm plate and infected with EV-C at a multiplicity of ca. 0.01. The cells were cultured in growth medium and transferred every 2 or 3 days. At each transfer, the growth medium was harvested for DNA polymerase activity, and 9/10 of the cells were used to prepare cellular DNA, whereas 1/10 was used to establish a new culture. The DNA preparations were digested with *Sac*I endonuclease and analyzed by Southern DNA transfer for the presence of free and integrated viral DNA. The endogenous virus from the Reaseheath line C is not cleaved by *Sac*I. Consequently, the free linear migrates as a single band with a molecular size of 7.5 kilobases (kb). Integrated EV-C DNA also remains uncleaved, and since integration occurs at multiple sites, it is visualized as a smear of hybridization to DNA fragments with sizes larger than 7.5 kb (13). The DNA polymerase activity detected in this experiment was indistinguishable from that observed in Fig. 4B. The analysis of the viral DNA present in Gr⁺ and Gr⁻ cells after infection by EV-C is presented in Fig. 5. The results demonstrate that significantly more linear viral DNA was synthesized in Gr⁺ than in Gr⁻ cells as has been reported (12). Further, linear viral DNA was detected one cell transfer sooner in the Gr⁺ infected cells. The presence of integrated endogenous virus in the Gr⁺ cell was seen at the time of the second transfer. Linear viral DNA appeared to be largely absent (or at least reduced 10-fold) in the cells at the time of preparation of the T₃ culture. In Gr⁻ cells, the linear viral DNA was not observed until the second culture transfer, whereas significant amounts of integrated viral DNA were not seen until the third transfer. By the fourth transfer, no significant amount of linear viral DNA was detected in the Gr⁻ cells. It should be noted that two- or threefold more integrated viral sequences were detected in Gr⁺ cells than in Gr⁻ cells.

TABLE 3. Expression of EV-C in clonal populations of infected Gr⁻ CEF^a

Clone	Virus titer (IU/ml) ^b	% Gr ⁻ CEF which produced virus ^c	Virus produced (IU) per infected cell ^d
1	10 ⁶	80	0.25
3	10 ⁴	80	0.0025
4	10 ²	2	0.003
5	5 × 10 ³	20	0.005
6	5 × 10 ³	20	0.005
7	10 ⁴	ND ^e	
8	10 ⁶	60	0.3
12	<10	ND	
13	<10	ND	

^a Clonal populations prepared in the experiment described in Table 2 were analyzed at the T₃ culture for production of infectious virus. The clone numbers correspond to those presented in Table 2.

^b Fresh growth medium was added (0.5 ml per 50-mm plate), harvested after 3 h from T₃ cultures, and titrated on Gr⁺ CEF.

^c Cells from the T₃ cultures were assayed in an infectious center assay on Gr⁺ CEF after the collection of culture fluids.

^d For this calculation it was assumed that there were 2.5 × 10⁶ cells per plate at the time of virus harvest.

^e ND, Not determined.

These results were confirmed by analysis with the restriction endonuclease *Hha*I. *Hha*I digestion of exogenously acquired RAV-0 DNA sequences produces two characteristic fragments of 1.35 and 0.65 kb that are readily detected after Southern DNA analysis (13, 14). These fragments are not produced by *Hha*I digestion of the endogenous viral loci, presumably because the sites are methylated. *Hha*I analysis revealed that there was only slightly more integrated EV-C DNA in Gr⁺ cells than in Gr⁻ cells (Fig. 6). These data indicate that the endogenous virus integrated with roughly equal efficiency in both permissive and nonpermissive cells. Further, the delay in the appearance of the viral linear DNA in Gr⁻ cells was in agreement with the rate of endogenous viral replication in these cells.

DISCUSSION

We used several different approaches to examine the replication of the chicken endogenous virus in nonpermissive chicken cells. The results of this study indicate that the principal difference between permissive and nonpermissive cells is the rate at which viral replication occurs after initial infection of the two types of cells. This conclusion is based upon three observations. First, there appears to be no difference in the efficiency with which EV-C establishes infection in permissive (line 15_B) and nonpermissive (SC) fibroblasts. In several experiments, equal numbers of infected clones of both cell types were isolated. This result indicates that a majority of the EV-C particles that can infect line 15_B cells can also adsorb to and replicate in a majority of the SC fibroblasts.

Second, EV-C-infected Gr⁻ cells produce virus particles, measured either by infectivity or DNA polymerase activity, at a rate that eventually equals that produced by infected Gr⁺ cells. This experiment has been repeated with several different virus isolates, using four different multiplicities of infection. The results demonstrate that, initially, viral replication proceeded more rapidly in Gr⁺ cells than in Gr⁻ cells. However, the amount of virus produced from Gr⁻ cells increased gradually with continued cell transfer until as

FIG. 4. Replication of EV-C and EV-C* in permissive (Gr^+) and nonpermissive (Gr^-) CEF. In 50-mm culture dishes, either [$15_B \times K(-)$ $\times K_{28}$ (Gr^+) CEF or SC (Gr^-) CEF were exposed to either EV-C or EV-C*. The cells were cultured in growth medium, and 1/10 of the cells were transferred every 2 or 3 days. Growth medium was harvested at each transfer to determine the amount of DNA polymerase activity present. The multiplicity of the infection was (A) 0.01 or (B) 0.00001. Background DNA polymerase activity present in uninfected Gr^+ cultures (x) was determined.

TABLE 4. Clonal analysis of the replication of EV-C and EV-C* in Gr^- CEF^a

Pheno- type	Production of DNA poly- merase activity			No. of EV- C-infected Gr^- clones	No. of EV- C*-infected Gr^- clones
	T ₃	T ₇	T ₁₁		
A	-	-	-	2	3
B	-	-	+	13	7
C	+/-	-	+	6	6
D	+	-	+	3	7
E	+	+	+	3	1

^a SC CEF were infected with either EV-C or EV-C* and cloned as in the experiment described in Table 2. The clones observed in this experiment were classified into phenotypes designated A, B, C, D, or E. The phenotypes of the clones are based upon the presence of detectable DNA polymerase in the growth medium harvested from T₃, T₇, or T₁₁ cultures. The designations +, +/-, and - are defined in Table 1, footnote b.

much virus was produced from the Gr^- cells as was produced from the Gr^+ cells.

Third, EV-C appears to integrate equally well in both Gr^+ and Gr^- CEF, if the infected cells are maintained in culture for sufficiently long periods of time. As observed by Hughes et al. (12), less linear viral DNA was detected during the first few days after infection of Gr^- cells. In the experiments described here, the difference was 10- to 50-fold. It is possible that the amount of DNA synthesis during the initial cycle of infection is equal in both cell types so that the difference detected in T₁ and T₂ cultures reflects the difference in the rate at which successive rounds of infection are established in the Gr^+ and Gr^- cells. T₁ and T₂ cultures underwent three and six cell cycles, respectively. Hughes et al. (12) reported a six- to eightfold reduction in viral DNA synthesis detected 12 h after infection of unsynchronized cells, which may or may not be the result of the decreased rate at which the endogenous virus infection spreads in the

FIG. 5. Analysis of free and integrated viral DNA present in cultures of Gr⁺ and Gr⁻ cells infected with EV-C. Gr⁺ and Gr⁻ CEF were infected with EV-C at a multiplicity of 0.01. The cells were cultured in growth medium and transferred at 2- to 3-day intervals. Total cellular DNA was prepared from cells at each transfer as described. The DNA was subjected to digestion with *SacI* restriction endonuclease and Southern DNA transfer analysis. EV-C DNA sequences were detected by hybridization with a RAV-0 DNA probe. The markers indicate the location of *ev-1* (9.5 kb), *ev-4* (8.8 kb), and a 7.5-kb DNA fragment. The DNA samples are from T₁ (lane 1), T₂ (lane 2), T₃ (lane 3), T₄ (lane 4), T₅ (lane 5), and T₆ (lane 6) cultures. (A) EV-C-infected Gr⁺ CEF. (B) EV-C-infected Gr⁻ CEF.

Gr⁻ cells. Despite the initial reduction in viral DNA synthesis observed in the Gr⁻ cells, successive transfer of the infected cells demonstrates that there is no inhibition in the accumulation of integrated EV-C DNA sequences.

This study, therefore, documents three observations that define the nonpermissive nature of the Gr⁻ cell. (i) The endogenous virus can adsorb to and infect Gr⁺ and Gr⁻ CEF with equal efficiency. (ii) Virus replication, as measured by the production of progeny virus, although greatly reduced initially, gradually increases to an unrestricted level in Gr⁻ CEF. (iii) Despite an initial reduction in the rate of DNA synthesis, there is a normal accumulation of integrated viral DNA sequences.

It should be noted that the replication of both RAV-2 and RAV-60, two exogenous viruses with subgroup B and E glycoproteins, respectively, was unrestricted in the Gr⁻ cells (data not shown). These viruses showed no initial delay in establishing infection in these cells, indicating that (i) exogenous viruses are not subject to the Gr⁻ restriction and (ii) a subgroup E glycoprotein does not per se constitute a sufficient condition for restricted growth. Further, both viruses were produced at approximately equal rates from cells permissive and nonpermissive for EV-C replication. It is important to point out that the replication of either RAV-2 or RAV-60 produces 10- to 20-fold more virus from either Gr⁺ or Gr⁻ CEF than does the replication of EV-C (data not shown) (21). This difference in the expression of these viruses is not associated with the genetic properties of the host cells but with genetic differences located in the 3' portions of their viral genomes (29). Whether the character-

istics of EV-C replication in nonpermissive cells are due to the 3' portion of the viral genome is unknown.

As already stated, the original intention of this study was, in part, to analyze the production of a variant virus capable of nonrestricted replication in Gr⁻ cells (21). Despite several attempts to isolate such a virus, no stable variants were generated during extended EV-C replication in SC fibroblasts. The efficient replication of EV-C that we observed after prolonged culture of infected Gr⁻ cells was not associated with the appearance and selection of a variant. The virus produced from these infected cells was indistinguishable from the original infecting virus. It seems most likely that the efficient replication of EV-C is the result of a gradual and progressive failure of the cellular restriction to inhibit virus replication. It is possible that the difference between the findings of this study and those published elsewhere (21) are due to differences in the properties of the SC and the K₂₈ fibroblast.

The data obtained from the analysis of clones of EV-C-infected Gr⁻ cells (Table 3) provide additional information that is helpful in understanding the progress of endogenous viral infection of Gr⁻ cells. This experiment identified clonal populations that, although they contained similar numbers of infected cells, produced virus at rates that differed by ca. 100-fold. This observation indicates that infection of a Gr⁻ fibroblast frequently produces a clonal population of cells of which the majority remain infected. Many of these clones, however, produced no more than 0.0025 to 0.005 infectious units per infected cell in 2 h. The experiment described in Table 2 demonstrates that as these clones are expanded the

FIG. 6. *Hha*I restriction endonuclease analysis of DNA from Gr^+ and Gr^- CEF infected with EV-C. The experiment described in the legend to Fig. 5 was further analyzed with *Hha*I restriction endonuclease. DNA from T_4 (lane 2) and T_5 (lane 3) cultures of EV-C-infected Gr^+ CEF and T_6 (lane 5) and T_7 (lane 6) cultures of EV-C-infected Gr^- CEF was digested with *Hha*I and analyzed by Southern transfer and hybridization to a RAV-0 DNA probe. Uncut DNA from the Gr^+ T_5 culture (lane 1) and the Gr^- T_7 (lane 7) culture was also analyzed in the absence of *Hha*I digestion to detect the presence of unintegrated viral DNA. Uninfected cell DNA (lane 4) containing *ev-1* was analyzed by *Hha*I digestion as a negative control. Markers indicate the location of the 1.35- and 0.65-kb DNA fragments.

rate of virus production increases to 0.2 to 0.3 infectious units per infected cell. It should be noted that, in the experiments with nonclonal populations of EV-C-infected Gr^- cells, the rate of virus production from fully infected cultures is ca. 0.5 infectious particles per infected cell in 2 h (assuming a half-life of ca. 2 h). Therefore, after continued replication in Gr^- cells, the endogenous virus can produce as much infectious progeny from Gr^- cells as from infected Gr^+ cells. The rate at which the virus spreads through cultures of uninfected Gr^- cells, however, is significantly slower than in Gr^+ cells. Further, our data demonstrate that clones of Gr^- cells containing a large proportion of infected cells (>20%) must be maintained in culture for extended periods of time before efficient replication of the virus can be detected. Based upon experiments with uncloned cells (Fig. 1 and 4) and populations of cloned cells (Tables 1 and 2), we estimate that an EV-C infection of Gr^- CEF must produce

between 10^7 and 10^8 infected cells before efficient endogenous virus replication is observed.

The results presented here and elsewhere (21) suggest that a step in the replication of the endogenous virus occurs efficiently in Gr^+ cells and inefficiently in Gr^- cells. It has been suggested that an early step, such as viral attachment involving the subgroup E envelope glycoprotein, is involved (12). Since our experiments established clonal populations by trypsinizing and replating cells 4 h after infection, the restriction of replication seems more likely to involve a postpenetration event.

Data presented elsewhere (12) suggest that viral DNA synthesis may be inhibited in Gr^- CEF. Such an inhibition may be the result of different rates of uncoating and polymerase activation or of DNA synthesis itself. Regardless of the step involved, it will be essential to ensure that any difference be measured by using experimental conditions that prevent a second cycle of infection. Such conditions will prevent the increased rate of replication of EV-C that occurs in Gr^+ CEF from distorting measurements of the initial rates. The replication of the endogenous virus may fail to proceed efficiently in Gr^- cells because an active integration intermediate is not formed or, alternatively, because the active intermediate is inactivated in the nonpermissive cell. Both these possibilities have been suggested as explanations for the *Fv-1* restriction that blocks replication of certain strains of murine leukemia virus (16–18).

In evaluating models that attempt to explain the Gr^- phenotype, it should be remembered that EV-C replication provides a stably infected population of cells that is, initially, inefficient in producing virus progeny. Continued replication of the virus appears to overcome the restriction. We are currently evaluating the hypothesis that endogenous virus replication in Gr^- cells consists of two phases. During the first phase, replication occurs in the absence of integration of viral DNA sequences. At this time, transcription of viral RNA proceeds inefficiently from unintegrated, infectious DNA intermediates. The second phase consists of a gradual accumulation of integrated sequences from which transcription of viral RNA can occur efficiently. With more efficient virus transcription, proceeding from integrated proviral sequences, and subsequent increased virus production, there would be an exponential increase in the production of integrated viral DNA. The virus produced from the integrated DNA sequences is not phenotypically altered and must repeat the inefficient cycle of virus replication after reinfection of the nonpermissive cells. It should be noted that replication of visna virus, a retrovirus that causes a slow infection of sheep and produces a non-neoplastic neurological degenerative disease, may proceed in the absence of viral DNA integration (8).

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