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Rous sarcoma virus infection of synchronized cells establishes provirus integration during S-phase DNA synthesis prior to cellular division

(colchicine arrest/viral activation/restriction endonucleases/Southern DNA transfer)

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ABSTRACT **Synchronized chicken embryo fibroblasts, prepared by addition of serum to stationary cells arrested in G_0, were exposed to the Prague strain of Rous sarcoma virus. At different times during the cell cycle, high molecular weight DNA was prepared from infected cells and examined for the presence of newly integrated viral DNA sequences. The results demonstrate that newly integrated viral sequences were first detected during S-phase DNA synthesis 9 hr after infection. The presence of colchicine prevented cellular division and delayed the appearance of progeny virus but it did not affect the appearance of viral specific DNA in the high molecular weight fraction of cellular DNA. Our results indicate that provirus integration, occuring during S-phase DNA synthesis, does not require cell division. Previous experiments have demonstrated that Rous sarcoma virus infection of chicken embryo fibroblasts requires cell division to initiate viral RNA synthesis and the production of progeny virus. The findings presented in this report support the hypothesis that division of the infected cell is required for an event that controls viral expression at the level of the integrated provirus.**

The replication of Rous sarcoma virus (RSV) proceeds through the formation and integration of a DNA copy of the viral genome, the provirus (1). Initiation of transcription of the provirus and the production of infectious virus require an initial division of the newly infected cell (2, 3). However, when cells are arrested in G_0 after the production of progeny RSV has already begun, both the transcription of viral RNA and the production of virus continue in the absence of further cell division (3, 4). The requirement for cell division, therefore, is not continuous and appears to be an event necessary for activation of the provirus (5).

Analysis of the early events in provirus formation has demonstrated that viral DNA synthesis is initiated after RSV infection of stationary cells (6). Examination of the viral DNA synthesized in these cells has revealed that it is incomplete and noninfectious (7). Complete synthesis of the provirus and its integration into cellular DNA has been shown to require an undefined host cell function(s) (8). To determine whether this host function(s) is related to the event(s) required for activation of the provirus, we analyzed the integration of the provirus in synchronized cells. The results presented below indicate that the complete provirus can be synthesized and integrated during S-phase DNA synthesis. Although the presence of colchicine inhibits the production of progeny virus, it has no effect on either the synthesis or integration of the provirus. It is likely, therefore, that the host function(s) required for the synthesis and integration of viral DNA is distinct from that required to activate the provirus. It seems that activation of the provirus depends on an event(s) that follows mitosis because colchicine-treated cells arrested in metaphase contain an inactive provirus.

EXPERIMENTAL PROCEDURES

Cells and Virus. Chicken embryo fibroblasts (CEF) were grown, in Dulbecco's modified Eagle's medium (Flow Laboratories, Rockville, MD) containing 10% tryptose phosphate broth (ET medium) and with calf serum (Sterile Systems, Logan, UT), in plastic tissue culture dishes (Nunc Plastics, Denmark). All experiments were carried out with cells derived from a single White Leghorn embryo (SPAFAS, Storrs, CT) that was C/E, avian leukosis virus-free, and contained only the *ev-1* locus (9). These cells were found to be negative for chicken helper factor and group-specific antigens (2, 10).

A single clone of the Prague strain of RSV, subgroup A (PR-RSV-A), originally obtained from J. Wyke, was used throughout this study. Production of PR-RSV-A progeny was determined by using a standard focus assay (11).

Preparation and Infection of Stationary Cells. Stationary CEF were prepared by plating 4×10^6 cells per 90-mm dish. Four hours after plating, the medium was changed to ET medium containing 0.5% calf serum, 1.0% horse serum (Flow), 20 mM Hepes (pH 8.0), and 2 mM glutamine and the cells were incubated at 38°C. Forty-eight hours after plating, <2% of the cells were labeled by exposure to [^{3}H]thymidine medium for 24 hr as described (4). After serum stimulation of these stationary cells, 85–95% of the nuclei were labeled with [^{3}H]thymidine (ref. 4; unpublished data). At this time, the cultures were changed to ET medium containing 10% calf serum. Three hours later, the medium was removed and, in a medium containing 10% calf serum, the cells were exposed to PR-RSV-A at a multiplicity of approximately 1–2 in an inoculum of 1 ml per plate. After 45 min at 38°C, the inoculum was removed and ET medium containing 10% calf serum added to the infected cells. Colchicine (Sigma) was added to some plates 5 hr after addition of serum (2 hr after infection) at a final concentration of 0.5 μM. The number of cells per culture dish and the rate of DNA synthesis were monitored during the cell cycle as described (4).

DNA Purification. High molecular weight DNA was purified from cells as described (12). PR-RSV-A DNA intermediates were prepared by exposing C/E CEF to PR-RSV-A for 3 consecutive hr at a multiplicity of approximately 3–4; the inoculum was changed each hour. Thirty-six hours after infection, the cells were used to prepare linear PR-RSV-A DNA as described (12).

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Abbreviations: RSV, Rous sarcoma virus; CEF, chicken embryo fibroblast; PR-RSV-A, subgroup A of Prague strain of RSV; kbp, kilobase pair(s).

Agarose Gel Electrophoresis, Southern DNA Transfer, and Hybridization. Gel electrophoresis was carried out in 1% agarose (Seakem)/60 mM Tris acetate 7.8/60 mM NaCl/6 mM EDTA. Samples were loaded in 10 mM Tris·HCl, 7.8/10 mM NaCl/1 mM EDTA/20% (wt/vol) sucrose/0.05% bromophenol blue. Restriction endonucleases *Bgl* II, *Eco*RI, *Hin*cII, and *Xba* I were purified and used as described (12). High molecular weight cellular DNA and unintegrated viral DNA intermediates were separated by agarose gel electrophoresis. Approximately 30–35 μg of uncut cellular DNA in a volume of 300–400 μl was electrophoresed on a 1% low-melt agarose (Bethesda Research Laboratories, Rockville, MD) tube gel (diameter 1.8 cm). Electrophoresis was continued until a 6.25×10^6 dalton marker, the *Hin*dIII fragment B of λ, had migrated approximately 1.6 cm.

Gel slices (1.5 mm) were dialyzed twice at 25°C for 15 min-periods against 1 ml of 10 mM Tris·HCl, 7.5/10 mM $MgCl_2$. The slices were pooled into three fractions: 0–0.6 mm, 0.6–1.2 mm, and 1.2–1.8 mm. The pooled fractions were melted at 68°C for 5 min, cooled to 37°C, digested to completion with *Hin*cII after the addition of 1 μg of λ DNA to each fraction, phenol extracted, and ethanol precipitated overnight at −20°C. After centrifugation at 30,000 × *g* for 10 min, the samples were resuspended and electrophoresed on 1% agarose slab gels. Ethidium bromide staining indicated that the λ DNA was recovered equally (80–90%) from all three fractions. The gels were prepared for Southern DNA transfer and the DNA on nitrocellulose filters was hybridized as described (12). PR-RSV-B 35S genomic RNA was degraded in 50 mM Na_2CO_3 for 18 min at 50°C and labeled, at the 5′-OH ends so generated, by polynucleotide kinase (P-L Biochemicals) and [γ-^{32}P]ATP (specific activity, ≈7500 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear). The specific activity of the viral RNA was approximately 1×10^8 cpm/μg. Initial hybridization studies using PR-RSV-A and PR-RSV-B [^{32}P]RNA produced identical patterns with *Hin*cII-digested PR-RSV-A DNA. Because PR-RSV-B 35S RNA is more easily obtained, it was used routinely during this study. Genomic 35S RNA isolated from the tdPRB strain of RSV was used as a probe containing all the viral genes except *src* to identify DNA fragments containing *src*-related sequences present in the *Hin*cII analysis of DNA from uninfected cells. Fuji RX film and DuPont Cronex Lightning Plus intensifiers were used for autoradiography at −80°C.

RESULTS

***Hin*cII Analysis of the *ev-1* Locus and the Free and Integrated Forms of PR-RSV-A DNA.** RSV infection of CEF usually results in the synthesis of a viral DNA intermediate. Studies on RSV and other retroviruses suggest that this process involves the insertion of the viral DNA into many sites within the cellular DNA (12–15). Consequently, the ends of different viral DNA molecules are joined to different cellular DNA sequences. Both the unintegrated viral DNA and the inserted provirus are colinear with the genomic viral RNA and terminally redundant (13, 15, 16). Restriction enzyme analysis of a population of cells containing integrated viral DNA molecules will detect only those viral DNA fragments common to a majority of the integration events. Consequently, fragments containing the ends of integrated RSV proviruses joined to different lengths of cellular DNA will not be detected. In contrast, DNA fragments derived from the internal portion of the provirus will be common to all integration events that lead to a colinear provirus. Therefore, in order to be able to monitor the integration of an RSV DNA intermediate, the internal fragments of the viral DNA must be used as markers. Furthermore, these fragments must be distinct from those DNA fragments derived from the endogenous loci present in the cell prior to infection.

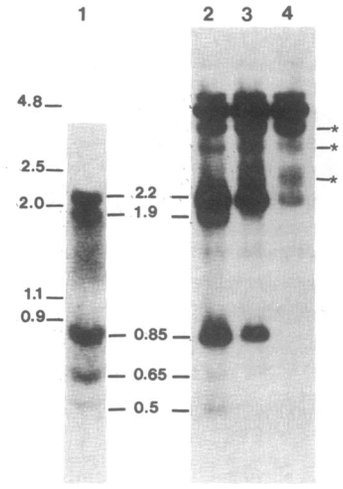

FIG. 1. *Hin*cII restriction endonuclease analysis of uninfected cell DNA, PR-RSV-A-infected cell DNA, and PR-RSV-A DNA. Samples of DNA were digested to completion with the restriction endonuclease *Hin*cII. The DNA fragments were analyzed by agarose gel electrophoresis, Southern DNA transfer, and hybridization to RSV[^{32}P]RNA. The autoradiogram shown represents a 72-hr exposure. The size markers were prepared from *Eco*RI (2.0, 2.5, and 4.8×10^6 daltons) and *Bam*HI (0.9 and 1.1×10^6 daltons) digestion of the *ev-1* and *ev-2* loci in DNA from line 100 C/BE cells (9, 12). The sizes for the PR-RSV-A DNA fragments, indicated in megadaltons, are based on their mobilities relative to those of the marker fragments. DNA fragments marked * are not detected after hybridization with tdPRB [^{32}P]RNA. Lanes: 1, linear PR-RSV-A DNA; 2, DNA prepared from CEF 36 hr after infection with PR-RSV-A; 3, DNA prepared from CEF 5 days after infection with PR-RSV-A; 4, DNA prepared from uninfected CEF.

In this investigation we used the site-specific restriction endonuclease *Hin*cII to analyze PR-RSV-A infection of CEF containing only the *ev-1* locus (9). *Hin*cII digestion of DNA from PR-RSV-A-infected cells revealed two new fragments not detected in a similar analysis of uninfected cells (Fig. 1). These *Hin*cII-specific fragments, 0.65 and 0.85 × 10^6 daltons, also were detected after *Hin*cII digestion of the linear form of PR-RSV-A DNA and have been located between 6.15 and 8.40 kilobase pairs (kbp) as shown on the PR-RSV-A linear map (Fig. 2).

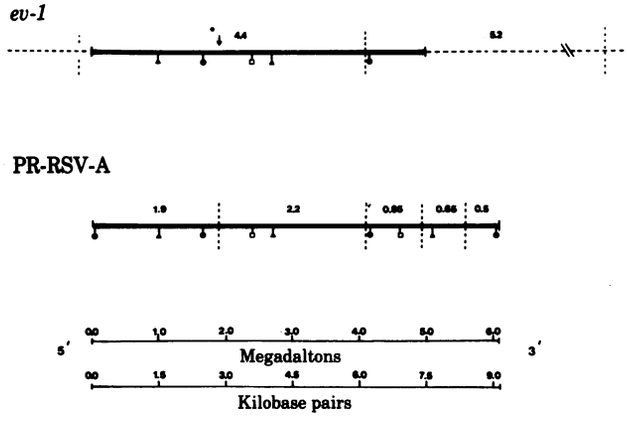

FIG. 2. *Hin*cII restriction endonuclease map of the *ev-1* locus and the linear PR-RSV-A intermediate. Cellular DNA at either end of the *ev-1* locus is designated by the horizontal dashed lines. *Hin*cII cleavage sites are located at positions marked by vertical dashed lines. The sizes of the *Hin*cII-specific DNA fragments are given in megadaltons. The *Hin*cII site in *ev-1* at 2.85 kbp (*) is apparently modified. Additional restriction enzyme cleavage sites are located as follows: ▲, *Bgl* II; ●, *Eco*RI; □, *Xba* I.

During the course of PR-RSV-A infection, two *Hin*cII-specific DNA fragments disappeared. Fragments of 0.5 and 1.9 × 10^6 daltons were observed after *Hin*cII digestion of cellular DNA prepared from cells 36 hr after infection and containing a mixture of both free and integrated PR-RSV-A sequences (Fig. 1). In contrast, these two fragments were not seen in an analysis of DNA prepared from cells 5 days after infection. These fragments have been mapped to the ends of the linear DNA intermediate (Fig. 2).

*Hin*cII digestion of the PR-RSV-A linear identified a fifth fragment, 2.2 × 10^6 daltons, located between 2.85 and 6.15 kbp on the PR-RSV-A map. An identical fragment frequently was observed as a minor product after *Hin*cII digestion of *ev-1* (Fig. 1; unpublished data). Under normal digestion conditions (1 unit/μg), *Hin*cII cleaved the *ev-1* locus only at 6.15 kbp, producing fragments of 5.2 and 4.4 × 10^6 daltons (Fig. 2; ref. 12; data not shown). Overdigestion with *Hin*cII, however, cleaved *ev-1* at a 2.85-kbp site–a site that is apparently modified in several endogenous loci—producing the 2.2 × 10^6 dalton fragment (data to be published elsewhere).

The data presented in Figs. 1 and 2 provide the information used to analyze the integration of PR-RSV-A. Two internal fragments, 0.65 and 0.85 × 10^6 daltons, can be used to monitor the synthesis and integration of PR-RSV-A specific DNA after infection of CEF containing only the *ev-1* locus. Two other fragments, 0.5 and 1.9 × 10^6 daltons, contain the 3′ and 5′ viral sequences of the linear intermediate and can be used to detect the presence of free viral DNA in DNA prepared from PR-RSV-A-infected cells. A fifth fragment, 2.2 × 10^6 daltons, is apparently identical to one derived from extensive *Hin*cII overdigestion of *ev-1*. This DNA fragment is usually a minor cleavage product of *ev-1* and so can be used as a third marker to monitor viral DNA synthesis and integration.

Analysis of Viral DNA Synthesis After RSV Infection of Synchronized Cells. CEF were synchronized by depletion of serum factors in the growth medium. Three hours after stimulation with serum, the cells were infected with PR-RSV-A. Colchicine was added to some of the cultures 2 hr after infection. The results obtained from monitoring the cell cycle and the production of progency virus are presented in Fig. 3. The results agree with those previously published (3, 4) showing (*i*) progeny virus is first detected at the time of cell division, (*ii*) colchicine, although not affecting S-phase DNA synthesis, prevents normal cell division, and (*iii*) colchicine delays virus production approximately 15 hr. Infected cells were harvested during each of the four distinct periods of the cell cycle, G_1, S, G_2, and mitosis, as well as during colchicine-induced metaphase arrest. Cells plated as infectious centers demonstrated that greater than 70% of the cells were infected 9 hr after infection (data not shown). Cells were also harvested 10 hr after the first cell division, at which time morphological transformation was evident in greater than 50% of the cells.

High molecular weight cellular DNA was prepared and examined for the presence of viral sequences. Samples of the different DNA preparations were first analyzed as high molecular weight DNA without restriction endonuclease digestion (Fig. 4*A*). Linear viral DNA was first detected 8 hr after infection. Little increase in the amount of viral DNA was detected during successive stages in the cell cycle, suggesting that the virus DNA was synthesized relatively synchronously. Furthermore, the addition of colchicine 2 hr after infection did not alter the amount of viral DNA synthesized as detected in metaphase arrest (17 hr after infection). *Hin*cII digestion of these DNA preparations reveal the five specific viral DNA fragments characteristic of the viral linear DNA described in Figs. 1 and 2 (Fig. 4*B*). The fragments were easily visualized in all the DNA samples except that from cells 3 hr after infection. There were three fragments that were not part of *ev-1* and that do not belong to the linear PR-RSV-A DNA. These fragments are not detected by using a tdPRB RNA probe and presumably contain *src*-related sequences (data not shown).

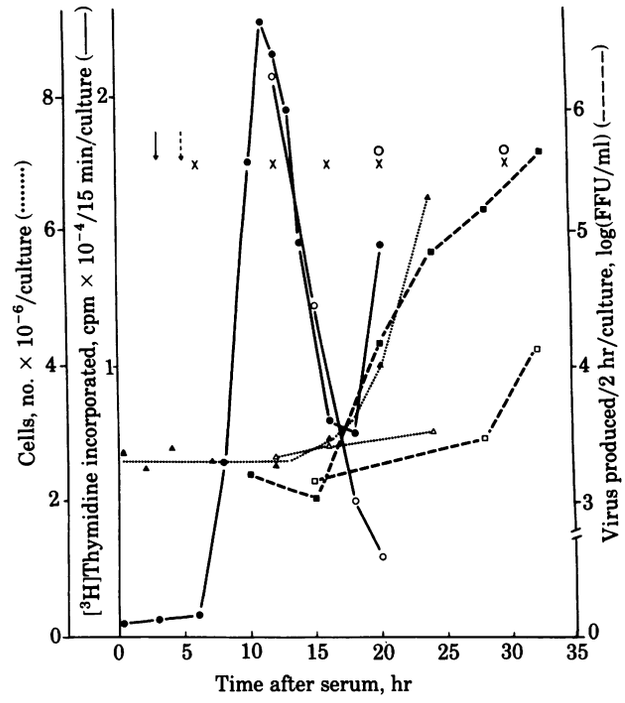

FIG. 3. PR-RSV-A infection of synchronized CEF. Cells were synchronized as described in the text. Three hours after addition of serum, the cells were exposed to PR-RSV-A (↓). Some cultures were treated with colchicine 5 hr after addition of serum (⇩). At different times, the rate of DNA synthesis (●, ○), the number of cells per culture (▲, △), and the virus produced during a 2-hr interval (■, □) were determined for untreated and colchicine-treated cultures, respectively. As indicated, untreated (×) and colchicine-treated (○) cells were harvested for preparation of DNA. FFU, Foci-forming units.

The *Hin*cII analysis of viral DNA synthesis after RSV infection of synchronized cells provides information concerning several points. Despite interruption of the cell cycle for the PR-RSV-A infection, the cell synchrony achieved distinguished the four major periods of the cell cycle. The infection could be monitored with sufficient sensitivity and specificity to detect viral DNA synthesis during the first round of infection. Viral DNA synthesis usually was not detected during the first 3–4 hr after infection—that is, during G_1. Five to 6 hr later, however, during S-phase DNA synthesis, viral DNA was easily visualized. It would appear that a majority of the viral DNA is synthesized during the period of S-phase DNA synthesis. As detected by using hybridization of DNA after the Southern transfer, the amount of viral DNA synthesized in colchicine-treated cells was not significantly different from that detected in untreated cells.

Analysis of RSV Provirus Integration in Synchronized Cells. To monitor the integration of the newly synthesized viral DNA, unintegrated viral DNA was removed from the high molecular weight cellular DNA by gel electrophoresis. DNA was electrophoresed on tube gels, fractionated, and digested with *Hin*cII. A sample gel displaying the electrophoresis of the *Hin*cII fragments in the second dimension is presented in Fig. 5. DNA was prepared, 17 hr after infection, from cells in colchicine-induced metaphase arrest. The DNA was fractionated so that unintegrated viral DNA electrophoresed approximately 1.5–1.6 cm in the first dimension while cellular DNA migrated no further

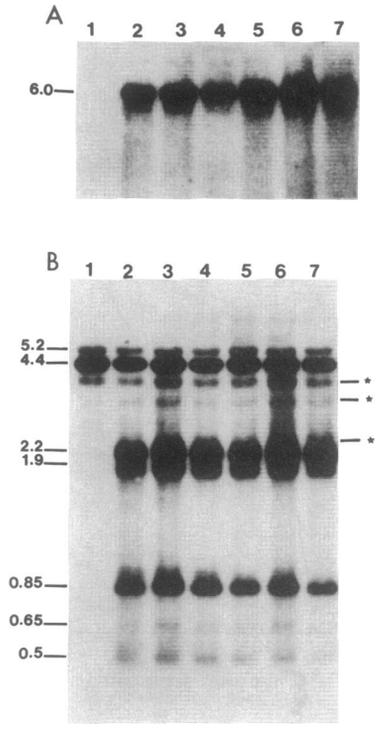

FIG. 4. Analysis of viral DNA present in different populations of synchronized PR-RSV-A-infected cells. DNA preparations from the cells harvested as described in Fig. 3 were analyzed by gel electrophoresis, Southern DNA transfer, and hybridization. DNA samples were analyzed either directly without restriction endonuclease digestion (*A*) or after digestion to completion with *Hin*cII (*B*). DNA was prepared from cells harvested at various times after infection. Lanes: 1, 3 hr; 2, 9 hr; 3, 12 hr; 4, 17 hr; 5, 17 hr and treated with colchicine; 6, 27 hr; 7, 27 hr and treated with colchicine. The autoradiogram shown represents a 48-hr exposure. The designated sizes (in megadaltons) were determined as in Fig. 1. The three bands marked * are not detected with a tdPRB probe.

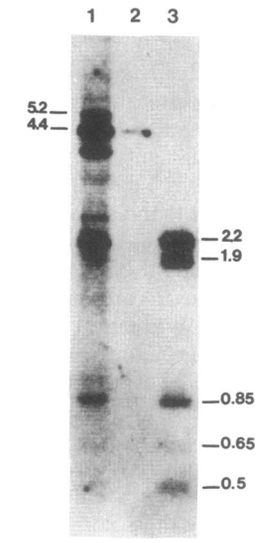

FIG. 5. Two-dimensional gel electrophoresis of DNA from PR-RSV-A-infected cells. DNA prepared from colchicine-treated cells harvested 17 hr after infection was fractionated on a 1% agarose tube gel. Then, the DNA was recovered, digested to completion with *Hin*cII, and analyzed by slab gel electrophoresis, Southern DNA transfer, and hybridization to RSV [^{32}P]RNA. The autoradiogram shown represents a 72-hr exposure. Lanes 1, 2, and 3 include 0–0.6 cm, 0.6–1.2 cm, and 1.2–1.8 cm of the tube gel, respectively. The 6.25×10^6 dalton λ DNA fragment used as a marker on the tube gel migrated 1.6 cm in this experiment.

than 0.6–0.7 cm. *Hin*cII analysis in the second dimension showed that five *Hin*cII fragments characteristic of linear PR-RSV-A DNA were present in fraction 3. Fraction 1, however, contained the fragments characteristic of *ev-1* plus the three *Hin*cII PR-RSV-A internal DNA fragments (Fig. 2), and so is identical to the PR-RSV-A-infected cell DNA (Fig. 1, lane 3). Significantly, although the *Hin*cII-specific internal fragments are present in the fraction containing the cellular DNA, the ends of PR-RSV-A linear are absent. This result demonstrates that PR-RSV-A sequences have fractionated (coelectrophoresed) with the high molecular weight cellular DNA. The results suggest that provirus integration occurs in synchronized cells prior to the completion of cellular division. Fig. 3 shows that the cells from which this DNA was prepared were not producing virus.

The two-dimensional gel analyses of all seven samples described in Fig. 3 and analyzed in Fig. 4 are presented in Fig. 6. Only the gel fractions containing the *Hin*cII digest of the cellular DNA extracted from fraction 1 are presented. The results show that unintegrated viral DNA appears to be absent from all these fractions because the ends of the linear are absent. The 0.85×10^6 dalton fragment is present in all samples. At both 9 and 12 hr after infection, a significant amount of this fragment was detected. Furthermore, there was an increase in the amount of the 2.2×10^6 dalton fragment derived from the internal portion of the PR-RSV-A linear. In some samples, the 0.65×10^6 fragment could be observed. It is clear that, although some viral DNA is integrated prior to the first cell division, some of the viral intermediate remains independent of association with host cell DNA (Fig. 5). The detection of integrated viral DNA in cells harvested 17 hr after infection, during mitosis, does not appear to be affected by the presence of colchicine. Ten hours later, 27 hr after infection, integrated viral DNA was again detected in metaphase-arrested cells. Significantly, metaphase-arrested cells studied 27 hr after infection contained integrated viral DNA but produced 1/75th to 1/50th as much

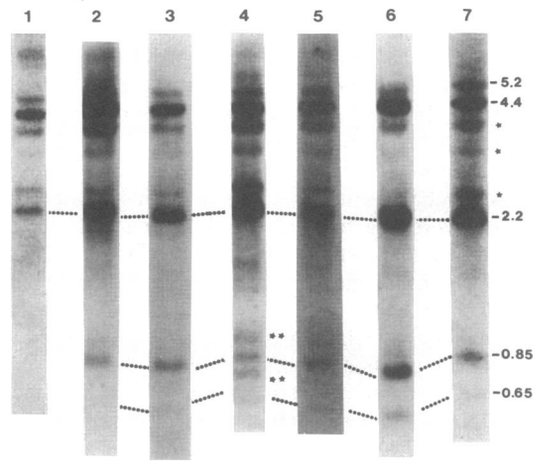

FIG. 6. Analysis of PR-RSV-A integration in synchronized CEF. The two-dimensional gel electrophoresis described in Fig. 5 was used to analyze all the DNA samples presented in Fig. 4. The autoradiograms pictured represent 72-hr exposures. Lanes: 1, 3 hr; 2, 9 hr; 3, 12 hr; 4, 17 hr; 5, 17 hr and treated with colchicine; 6, 27 hr; 7, 27 hr and treated with colchicine. The three bands marked * are those previously identified by using a tdPRB probe as described in Fig. 1. The two bands marked ** are observed after *Hin*cII overdigestion of DNA from uninfected cells but are not detected by using a tdPRB probe.

virus as control untreated cells. Colchicine does not inhibit the synthesis of either viral-specific RNA or progeny virus when added after virus production has begun (4, 17). It would appear, therefore, that the cell division required for viral expression is required not for integration of the provirus but rather for some event that controls its expression after integration.

DISCUSSION

Several investigations have demonstrated that RSV-infected cells must undergo division after infection, prior to the production of infectious progeny (2, 3, 5). Subsequent to these investigations, two independent studies demonstrated that the synthesis of viral DNA is incomplete after infection of stationary cells (7, 8). Because much of the work defining the requirement for cell division for activation of the RSV infection utilized infection of stationary cells, it was suggested that the dependency of RSV infection on cell cycle could be attributed to a need for host functions to complete the synthesis and integration of the provirus (8). The results presented here do not support that suggestion. Our experiments indicate that, after infection of synchronized cells, the synthesis and integration of RSV DNA occur primarily during the period of S-phase cellular DNA synthesis. In spite of the integration of a complete provirus during S-phase, no viral expression was observed prior to the first mitosis. Similarly, although integration of a complete provirus occurs in colchicine-treated cells, viral expression is delayed for 10–15 hr. These results indicate that the requirement for a cell division after RSV infection does not involve a requirement for synthesis and integration of the viral DNA. It appears, rather, that cell division is necessary for an event(s) that is required for activation of the integrated provirus.

In a separate study, the nature of the required event(s) was further examined by using cells infected with two distinct viruses. The presence of an active provirus within a cell did not eliminate the need for a subsequent cell division to activate the provirus formed after superinfection by a second virus. It appears, therefore, that the event(s) is specific for each provirus and therefore "*cis*-acting" in character (17).

Finally, colchicine only inhibits the synthesis of progeny for a period of approximately 10–15 hr. The appearance of virus at this time occurs in the absence of any detectable cell division. However, prolonged exposure of cells to colchicine does result in the formation of "mininuclei" within treated cells. It is curious that the maturation of progeny virus coincides with the appearance of these mininuclei because the formation of mininuclei is associated with decondensation of the chromosomes within newly formed nuclear membranes, thus providing nuclear division in the absence of cytokinesis (18). It is possible that the activation of the provirus, associated with the initiation of transcription (3), requires the replication of the integrated sequences prior to the formation of a active transcriptional complex.

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