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Variations in Integration Site of Avian Oncornaviruses in Different Hosts

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We examined the integration site of avian oncornaviruses in the genome of different hosts with respect to the repetitive frequency of the cellular DNA sequences adjacent to the integrated proviral DNA. The following systems were studied: avian sarcoma virus (B-77) and avian leukosis virus (Rous-associated virus-61) in cultured duck embryonic cells and B-77 in cultured mouse 3T3 cells. These systems represent different host responses to viral infection, i.e., one in which both cellular transformation and viral replication occur (B-77-infected duck cells), one in which viral replication, but not transformation, occurs (Rous-associated virus-61-infected duck cells), and one in which transformation, but not viral replication, occurs (B-77-infected 3T3 cells). Two sequential hybridizations were used. First, large denatured DNA fragments (2.8×10^6 daltons) were reassociated to different C_0t (mole · seconds per liter) values. Next, DNA remaining single stranded at different C_0t values was isolated by hydroxylapatite column chromatography, immobilized on nitrocellulose filters, and hybridized with an excess of ^3H-labeled 35S viral RNA to titrate the concentration of proviral DNA. Results show that B-77 sarcoma virus and Rous-associated virus-61 integrate in the unique region of duck DNA, whereas B-77 proviral DNA is associated with both repeated and unique host DNA sequences in transformed mouse 3T3 cells.

The DNA replicative intermediate (provirus) of avian oncornaviruses integrates into the host chromosomal DNA (13, 22, 23). Since subsequent expression of viral information may depend upon the location of the integration site in cellular DNA (14, 18), two basic questions present themselves: (i) determination of whether or not the integration of proviral DNA is site specific, and (ii) identification of the provirus site in relation to some known markers of host chromosomal DNA.

The localization of genes in eukaryotic cells is, at present, difficult because of the large complexity of the genome and the paucity of characterized cellular markers. However, eukaryotic DNA is composed of sequences whose frequency range from 10^0 to 10^6 times per haploid genome (4), and the proviral DNA can be localized with reference to the frequency of adjacent cellular sequences (8). Previously, we demonstrated that, in normal chicken cells, the endogenous proviral DNA resides in a region of cellular DNA that is reiterated between 1,000 to 2,000 times per haploid chicken genome (8, 9) and that, in chicken leukemic myeloblasts induced by avian myeloblastosis virus (AMV), the AMV provirus appears to be integrated in tandem with the endogenous proviral DNA (18).

In this report, we have attempted to localize, by a similar approach, the site of integration of B-77 avian sarcoma virus and of a Rous associated virus (RAV-61) in permissive cells (duck embryonic fibroblasts) and in nonpermissive cells (mouse 3T3 cells). Different modes of viral infection are represented by these virus-cell systems: (i) in B-77-infected duck cells, both viral production and transformation occur; (ii) in RAV-61-infected duck cells, virus production is observed in the absence of cellular transformation; and (iii) in B-77-infected mouse 3T3 cells, transformation is observed in the absence of virus production. In addition, the genome of uninfected duck cells or mouse cells does not contain sequences that hybridize significantly with the avian viruses used in this study (21, 23). Furthermore, the genome of birds differs significantly from that of mice in that 15 to 20% of the avian DNA consists of repeated DNA, whereas 30 to 40% of the murine DNA consists of reiterated DNA (8, 10). The genomic localization of the proviral DNA was carried out in the

following manner. DNA isolated from infected cells consisted of fragments sufficiently large to contain both viral and contiguous cellular sequences. The fragments were denatured and fractionated according to their reassociation rates using hydroxylapatite chromatography. Finally, the concentration of viral sequences in the DNA that remained single stranded in each fraction was quantitated by hybridization of the DNA immobilized on nitrocellulose filters with an excess of the appropriate 35*S* viral RNA (8, 18).

The results indicate that: (i) in duck embryonic fibroblasts infected with B-77 or RAV-61 virus, the viral DNA is primarily associated with unique cellular DNA, and (ii) in nonpermissive B-77-transformed mouse 3T3 cells, approximately 50% of the viral DNA appears to be associated with repeated DNA, and the rest is probably associated with unique cellular DNA.

MATERIALS AND METHODS

Viruses and cells. B-77 avian sarcoma virus and RAV-61 were kindly provided by H. Hanafusa of Rockefeller University. Duck embryonic fibroblasts were prepared from fertile eggs bought from Ward Duck Co., La Puente, Calif. Cloned Swiss-Webster mouse 3T3 cells were kindly provided by H. Herschman of the University of California, Los Angeles. Most duck and mouse cells were transformed within 3 days after infection with B-77 sarcoma virus, and all the cells appeared transformed when harvested approximately 3 weeks later after six subculture passages. The duck cells infected with RAV-61 were not transformed when DNA was extracted 3 weeks after infection. The general descriptions of the methods for culturing cells and propagating viruses have been published (1, 16). All cultured cells were tested for the absence of congenital viremia before use (2).

Viral RNA. The isolation of ^{3}H-labeled 70*S* viral RNA from purified virions and of ^{3}H-labeled 35*S* RNA subunits from ^{3}H-labeled 70*S* RNA after melting at 80°C for 3 min in 10^{-2} M Tris-hydrochloride (pH 7.4) and 10^{-3} M EDTA have been published (18).

Preparation of DNA. The method for preparing high-molecular-weight DNA has been described (8). Briefly, the infected cells were gently lysed in a Dounce homogenizer in reticulocyte standard buffer (0.01 M NaCl plus 0.01 M Tris-hydrochloride [pH 7.4] plus 0.003 M $MgCl_2$). Nuclei were pelleted, washed, and resuspended in SSC buffer (1× SSC = 0.15 M NaCl plus 0.015 M Na citrate) and 0.2% sodium dodecyl sulfate. RNase A and RNase T_1 were added at 100 μg/ml and 50 U/ml, respectively, and incubated for 12 h at 37°C. Predigested Pronase was then added to a concentration of 2 mg/ml and incubated for 12 h at 37°C. This solution was extracted with chloroform/isoamyl alcohol mixture (24:1) and dialyzed through 10 changes of SSC. DNA prepared in this manner had an average molecular weight greater than 12×10^6. The DNA was then sheared in a Waring blender at 0°C for 5 min to yield DNA fragments that had an average size of 19 to 21*S*. The size was determined by velocity sedimentation through a linear 5 to 20% alkaline sucrose gradient (0.3 N NaOH, 0.5 M NaCl, and 0.01 M EDTA) at 30,000 rpm for 13.5 h at 4°C in an SW40 rotor using phage φX174 DNA as an 18*S* marker.

DNA reassociation. The fragmented DNA was boiled for 5 min and allowed to reassociate at 60°C in phosphate buffer (PB; pH 6.8) (4). All C_0t values were standardized to 0.12 M PB to correct for variations in salt concentration needed to slow or accelerate individual reactions (8). The reassociation reaction was stopped by dilution to 0.01 M salt concentration. The samples were stored at 4°C and then passed through hydroxylapatite columns (DNA-grade Bio-Gel HTP, Bio-Rad) at 60°C. Single-stranded DNA was eluted with 0.17 M PB, and double-stranded DNA was eluted with 0.4 M PB. DNA concentrations were determined by measuring absorbance at 260 nm, and then each fraction was exhaustively dialyzed against 0.1× SSC.

DNA-RNA hybridization. The DNA remaining single stranded at each C_0t value was denatured and immobilized on nitrocellulose Millipore filters (0.45-μm pore size) as previously described (3). All filters were then hybridized with an excess (8×10^5 cpm/ml) of the appropriate ^{3}H-labeled 35*S* viral RNA (specific activity: 1.4×10^6 cpm/μg) as reported (3). ^{3}H-labeled viral RNA hybridized to single-stranded DNA obtained either by fractionating C_0t 0 DNA (i.e., DNA that was immersed in an ice bath immediately after denaturation and fractionated on hydroxylapatite) or from unfractionated denatured DNA represented the total concentration of viral DNA.

RESULTS

Size of DNA fragments from duck and mouse cells. The size of DNA fragments from RAV-61- or B-77-infected duck cells or from B-77-infected mouse cells was determined by alkaline sucrose gradient velocity sedimentation using ^{3}H-labeled φX174 circular single-stranded DNA as a marker (Fig. 1). All three DNAs had an average fragment size of 2.8 to 3.0 $\times 10^6$ daltons in single-stranded form, approximately equivalent to the size of one provirus.

Reassociation kinetics of DNA from infected duck or mouse cells. The reassociation kinetics of DNA fragments with an average size of 20*S* (2.8×10^6 daltons) from duck embryonic fibroblasts infected with RAV-61 (closed circles) or with B77 virus (open circles) are shown in Fig. 2. The large size of the fragments is responsible for the large percentage of DNA reassociated at C_0t 0 (~20%) and C_0t 100 (~40%).

The reassociation kinetics of mouse 3T3 DNA fragments with an average size of 20*S* (2.8×10^6 daltons) show that the proportion of DNA that renatures rapidly is larger than in duck DNA,

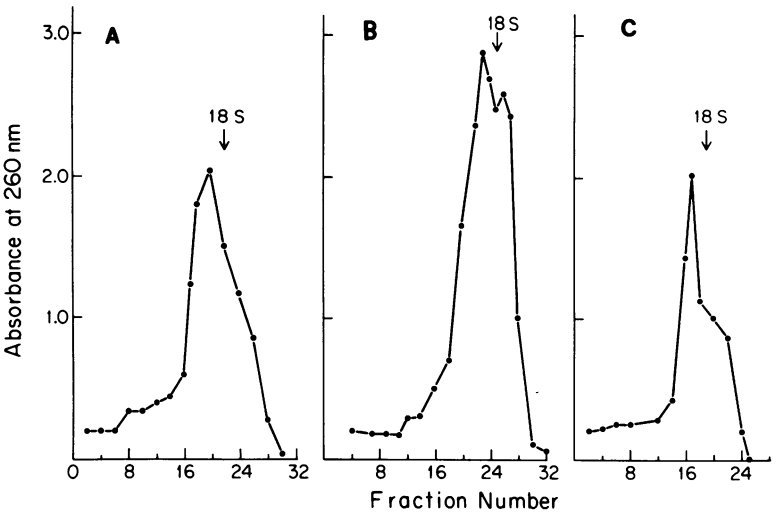

FIG. 1. *Alkaline sucrose velocity sedimentation profiles of DNA fragments from RAV-61-infected duck embryonic fibroblasts (A), B-77-infected duck embryonic fibroblasts (B), and B-77-infected mouse 3T3 cells (C). Between 100 and 200 μg of DNA was layered on a 5% to 20% sucrose gradient containing 0.3 N NaOH, 0.5 M NaCl, and 0.01 M EDTA in an SW40 rotor cellulose nitrate tube and sedimented at 30,000 rpm for 13.5 h at 4°C. Fractions were collected from the bottom of the tube, and absorbance of each fraction at 260 nm was measured in a Beckman spectrophotometer. ^{3}H-labeled φX-174 DNA was cosedimented as marker.*

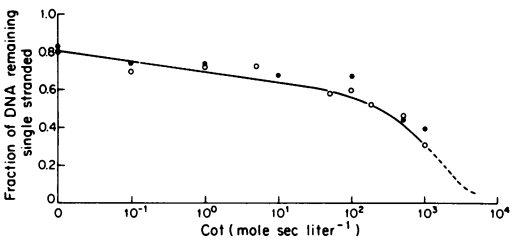

FIG. 2. *Kinetics of reassociation of duck DNA. High-molecular-weight duck DNA extracted from duck embryonic cells infected with RAV-61 (●), or B-77 virus (○) was sheared to an average size of 20S (2.8 × 10^6 daltons). The DNA was denatured and permitted to renature at 60°C. Samples were withdrawn at different time intervals, diluted in 0.01 M PB (pH 6.8), and kept on ice until passed through a column of hydroxylapatite at 60°C. The column was washed with 0.01 M PB, and then single-stranded DNA was eluted with five bed volumes of 0.17 M PB. Finally, the double-stranded DNA was eluted with an equal amount of 0.4 M PB. The concentration of single-stranded DNA was calculated from A_{260}.*

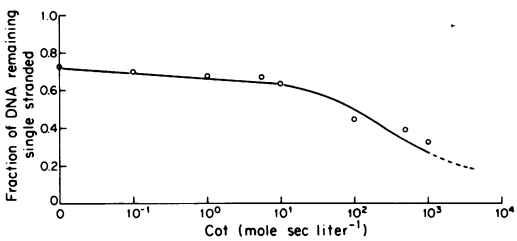

FIG. 3. *Kinetics of reassociation of mouse 3T3 DNA. High-molecular-weight mouse DNA extracted from mouse 3T3 cells infected with B-77 virus was sheared to an average size of 20S (2.8 × 10^6 daltons), denatured, and its kinetics of reassociation were analyzed as in Fig. 1.*

in accordance with the different arrangement and quantity of reiterated DNA sequences in the two DNAs (Fig. 3). Approximately 30% of the mouse DNA is renatured as C_0t 0 and 50% is renatured at C_0t 100.

A potential artifact that could occur during reassociation of large DNA fragments is the degradation of the fragments upon incubation at 60°C. If present, this artifact would slow down the renaturation rate at the higher C_0t values. We checked for the absence of degradation by determining the size of a test sample DNA (leukemic chicken myeloblast DNA) after incubation to various C_0t values under experimental conditions identical to those used in this study. As shown in Fig. 4, DNA fragments with an average sedimentation coefficient of 14S did not show a decrease in size after being reassociated to C_0t values as high as 10^3 mol·s/liter. The lower absorbances at 260 nm (A_{260}) in the peaks of Fig. 4D and E do not represent a lower recovery at higher C_0t values. The A_{260} values of the various peaks cannot be directly compared, because they have not been corrected for variations in dilution before the absorbance

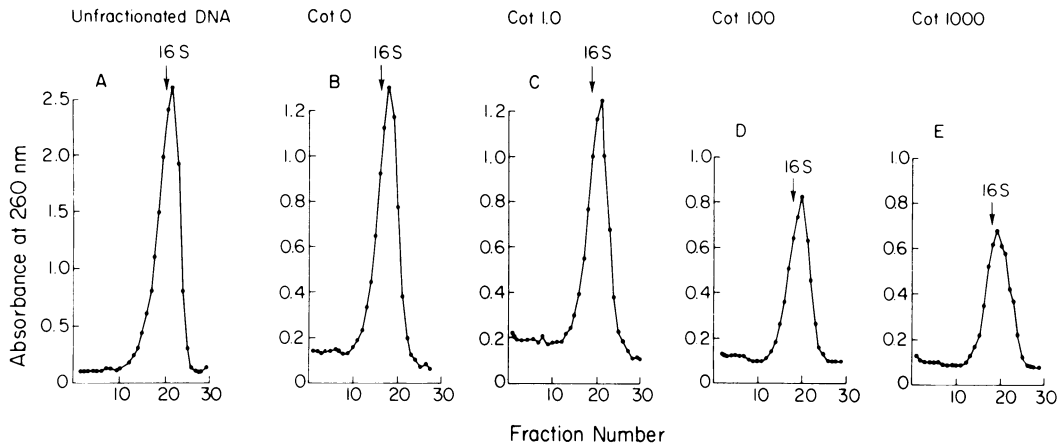

FIG. 4. *Alkaline sucrose velocity sedimentation analysis of DNA fragments renatured to different C_0t values. DNA fragments of approximately 14S were denatured and reassociated to different C_0t values. They were then sedimented in a 5% to 20% sucrose gradient containing 0.3 M NaOH, 0.5 M NaCl, and 0.01 M EDTA in an SW40 rotor cellulose nitrate tube at 27,000 rpm for 15 h at 4°C. Fractions were collected from the bottom of the tube, and the A_{260} of each fraction was measured in a Beckman spectrophotometer. ^{3}H-labeled M-13 DNA was cosedimented as 16S marker. (A) Untreated DNA fragments. (B)-(E) Denatured DNA fragments after reassociation to varying C_0t values: (B) C_0t 0, (C) C_0t 1, (D) C_0t 100, (E) C_0t 1,000.*

measurements.

Kinetics of renaturation of B-77 and RAV-61 proviral DNA segments integrated into DNA from infected duck cells. Figure 5 illustrates schematically two different models of viral DNA integration and the types of fragments that could be generated by shearing of the DNA. Only the region of cellular DNA contiguous to proviral DNA is shown in the diagram. Model 1 presents the model of viral integration involving a single 35S proviral DNA (3.0×10^6 daltons) that can be integrated in three different manners: (i) between two reiterated (R) regions, i.e., both X and Y equal R, (ii) between one reiterated and one unique (U) region, e.g., X = R and Y = U, or (iii) between two unique regions, i.e., both X and Y equal U. According to this model, shearing of the DNA to an average fragment size of 20S (2.8×10^6 daltons) would give rise to two average populations of fragments: one consisting of half the proviral DNA and adjacent cellular region X and the other consisting of the other half of proviral DNA and cellular region Y. Since the viral DNA sequences themselves are present as only one to three copies per haploid genome (8, 24), the proviral DNA reassociation will be influenced by the adjacent cellular sequences. There are three possibilities: (i) if both X and Y are reiterated, 100% of the viral sequences will be present in the partially double-stranded DNA fraction (as scored by hydroxylapatite) at early C_0t values; (ii) if the proviral DNA is inserted in the unique region of host DNA (i.e., both X and Y are unique), all the proviral DNA should reassociate as unique DNA and will be absent from the partially double-stranded DNA fraction at low C_0t values; (iii) if X is reiterated and Y is unique, or vice versa, 50% of the proviral DNA should be present in the double-stranded DNA fraction, and the other 50% should be found in the single-stranded fraction at low C_0t values.

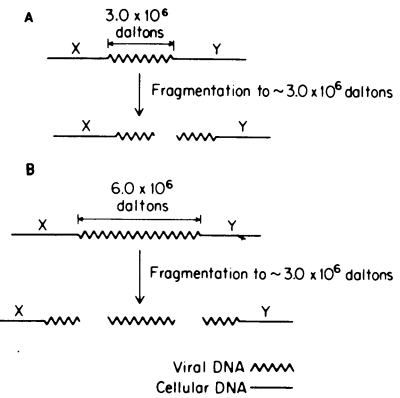

FIG. 5. *Schematic representation of two models of viral DNA integration. (A) Model 1, (B) model 2. The figure represents the fragmentation of proviral DNA and adjacent cellular DNA that occurs when high-molecular-weight DNA is sheared to a size of ~3.0 × 10⁶ daltons.*

Model 2 depicts the situation in which two proviruses are integrated in tandem; i.e., the size of the integrated proviral DNA is 6×10^6

daltons. Shearing into an average fragment size of approximately 3×10^6 daltons would then lead to three average populations of fragments containing proviral DNA. The two end fragments representing 50% of the proviral DNA would be associated with cellular DNA sequences, whereas the internal fragment would consist entirely of viral DNA (50% of total). The internal fragment would reassociate as unique DNA, whereas the two end fragments would reassociate according to the reiteration frequency of the adjacent cellular sequences. If both X and Y are reiterated, 50% of the proviral DNA will be present in the partially double-stranded DNA fraction at low C_0t values, whereas if X and Y are unique, all of the proviral DNA will be present in the single-stranded DNA fraction at low C_0t values. If X is reiterated and Y is unique, or vice versa, 25% of viral DNA should be associated with fragments that reassociate rapidly.

The kinetics of renaturation of viral DNA sequences from duck embryo fibroblasts infected with RAV-61 or with B-77 are shown in Fig. 6. The solid lines marked 1, 2, 3, and 4 represent theoretical curves corresponding to the different models of integration discussed above: Curve 1 corresponds to model 1 with both X and Y reiterated; curve 2 corresponds either to model 1 with X or Y reiterated or to model 2 with both X and Y reiterated; curve 3 corresponds to model 2 with either X or Y reiterated; and curve 4 corresponds to either model 1 or model 2 with both X and Y unique. The solid circles represent the kinetics of renaturation of RAV-61 DNA sequences, and the open circles show the renaturation of B-77 DNA sequences. In both cases, it appears that the proviral DNA sequences renatured according to curve 4; i.e., all the proviral sequences are located on fragments that reassociate as unique DNA. Some viral DNA is not integrated on fragments that reanneal at very low C_0t values, because all the proviral DNA is present in the single-stranded DNA fraction obtained by hydroxylapatite chromatography of C_0t 0 DNA (see Materials and Methods). These results suggest that the sites of provirus integration of RAV-61 and of B-77 sarcoma virus are located in unique duck DNA. These findings are in agreement with the conclusion reached by a different approach for the integration of B-77 proviral DNA in duck DNA (21). However, it is not possible to distinguish between models 1 and 2, i.e., whether there is one or two proviruses per integration site.

Since the proviral sequences by themselves should renature as unique DNA, the experimental data would also be consistent with the existence of the proviral DNA in a nonintegrated form. It has previously been shown that in permissive (duck) as well as in nonpermissive (BALB/C-3T3) host cells, the B-77 proviral DNA is integrated into the host DNA soon after infection (23), but can also remain in free form for as long as several weeks after infection (24). In the latter case, the unintegrated viral DNA is present exclusively in the cytoplasm (24). The DNA used in our experiments was isolated from cell nuclei excluding proviral DNA that may be present in the cytoplasm. Also, we did not detect free proviral DNA in the Hirt supernatant fluid from RAV-61- or B-77-infected duck cells after six cell culture passages (about 3 weeks after infection) when the cellular DNA was extracted for these experiments (unpublished data).

The fact that B-77 virus preparations may contain an excess of nontransforming (td) virus does not invalidate our conclusions. All the cells were transformed when DNA was extracted, and therefore each cell genome must have contained at least one integrated transforming B-77 provirus. Since the total number of integrated transforming and nontransforming proviruses is two to three per haploid cell genome (18, 24), at least 25% of the integrated proviral DNA represents a transforming viral

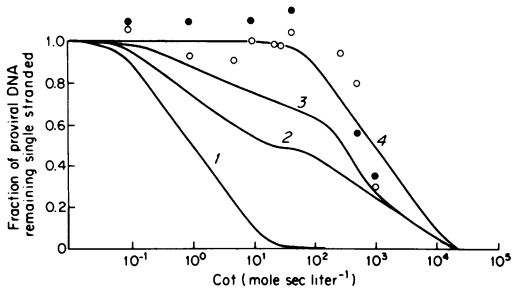

FIG. 6. *Kinetics of renaturation of viral sequences in DNA from infected duck embryonic fibroblasts. Single-stranded DNA was prepared as in Fig. 1. The samples were dialyzed against 0.1 × SSC, denatured, and immobilized on nitrocellulose filters as described earlier (3). Hybridization of duck DNA infected with RAV-61 (●) was performed with purified ^{3}H-labeled 35S RAV-61 RNA (0.8×10^6 cpm/ml; specific activity, 1.4×10^6 cpm/μg). Similarly, B-77-infected duck DNA was hybridized with purified ^{3}H-labeled 35S B-77 RNA (0.8×10^6 cpm/ml; specific activity, 1.4×10^6 cpm/μg) (○). The fraction of proviral DNA remaining single stranded at different C_0t values was calculated from the ratio of viral RNA counts per minute hybridized per 100 μg of single-stranded DNA to counts per minute hybridized per 100 μg of C_0t 0 DNA.*

genome. Our experimental procedure is sensitive enough to detect that proportion of fast-reassociating viral DNA if it existed. The presence of an excess of td viral RNA in our H^3-labeled viral RNA probe would not affect the results, since the transforming region represents only approximately 10% of the viral genome.

Kinetics of renaturation of B-77 proviral DNA sequences integrated into DNA from transformed mouse cells. Figure 7 shows the kinetics of renaturation of B-77 proviral sequences present on DNA fragments with an average size of about 3.0×10^6 daltons obtained from B-77-transformed mouse 3T3 cells. The experimental points (solid circles) give the best fit to theoretical curve 2, indicating that about 50% of the proviral sequences are located on fragments that renature rapidly with a $C_0t_{1/2}$ of approximately 10^0 and that the other 50% are located on fragments that renature as unique DNA with a $C_0t_{1/2}$ of approximately 10^3. The fraction of viral DNA in fragments smaller or larger than 3×10^6 daltons is not large enough to significantly alter our findings. From Fig. 1C, it can be seen that approximately 25% of the mouse DNA is in fragments that have a size of 0.5×10^6 to 1.5×10^6 daltons. Consequently, 15% or less of the proviral DNA should be on fragments that do not contain adjacent cellular DNA and should reassociate as nonrepeated DNA. Also, only approximately 15% of the proviral DNA should be present on fragments twice as large, or larger than, the provirus. It appears, therefore, that the B-77 provirus is integrated either as model 1 (Fig. 5) with one end adjacent to reiterated cellular sequences and the other end adjacent to unique cellular sequences or, as in model 2, with both ends adjacent to reiterated cellular sequences. A third possibility is that the B-77 provirus is randomly integrated, one copy per site, in the mouse genome, since approximately 50% of mouse DNA renatures by a C_0t of 100, and the other 50% renatures as unique DNA.

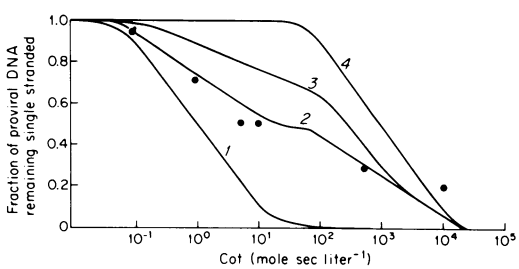

FIG. 7. *Kinetics of renaturation of viral sequences in DNA from B-77 transformed mouse 3T3 cells. Single-stranded DNA was prepared as in Fig. 1 and processed as in Fig. 4. The single-stranded DNA was hybridized with purified ^{3}H-labeled 35S B-77 RNA (0.8×10^6 cpm/ml; specific activity, 1.4×10^6 cpm/μg).*

DISCUSSION

In eukaryotic DNA, repetitive sequences are interspersed among unique DNA (4, 6, 12). The average length of the reiterated and unique DNA varies among different animal species (6, 12). Since at least 60% of duck DNA sheared into large fragments of approximately 3.0×10^6 daltons renature as unique DNA (Fig. 2), the interspersion distance between reiterated duck DNA sequences appears to be at least 20 kilobases. This general pattern is similar to the organization of the chicken genome (8; K. Som, M. N. Dastoor, M. Shoyab, and M. A. Baluda, unpublished data).

The reassociation pattern of denatured fragments that contain proviral DNA from duck cells infected with either an avian sarcoma virus or an avian leukosis virus indicates that the viral DNA is not integrated near reiterated DNA sequences. This would not be correct if there were more than six copies of the provirus integrated end-to-end. However, existing evidence suggests that it is not so (21). The presence of fragments that are larger or smaller than the average size of 3×10^6 daltons in the duck DNA preparation does not affect our conclusions. The larger fragments would be even more likely to contain reiterated cellular DNA sequences if they were located near the integration site and they would have accelerated the reassociation of viral DNA at low C_0t values. With smaller DNA fragments, proviral DNA would renature as unique DNA even if it were integrated next to repeated cellular sequences. However, less than 20% of the DNA is in fragments that have a size equal to or smaller than half that of the provirus; i.e., 1.5×10^6 daltons and less than 5% of the DNA is in fragments that have a size of 0.2×10^6 daltons or smaller. Therefore, less than 15% of the proviral DNA would be expected to reassociate as unique DNA due to its small size alone.

Since infected duck cells are permissive for viral replication and transformation, it appears that the proviral genes can be expressed, despite the fact that repetitive DNA sequences are not adjacent to them. Thus, viral gene expression in infected duck cells differs from the transcription model proposed for the sea urchin embryonic genome in which each single copy DNA sequence appears to be adjacent to repetitive sequences (7).

In mouse 3T3 cells infected with B-77 virus, the situation is different in that viral DNA sequences are associated with both unique and

reiterated cellular sequences. Unfortunately, we are unable with this method to propose an unambiguous arrangement of the proviral genes in the mouse genome because the number of provirus copies per integration site is unknown, and because the interspersion pattern of reiterated and single-copy DNA in the murine genome resembles that of *Xenopus* (6, 10).

It appears that the proviral DNA of avian oncornaviruses can integrate into host DNA at sites that differ in sequence reiteration frequency. The endogenous provirus in normal chicken cells and the exogenous avian myeloblastosis provirus in leukemic chicken cells are integrated very close, or adjacent, to reiterated host DNA sequences (8, 18). In duck embryonic fibroblasts, the avian leukosis virus (RAV-61) and B-77 sarcoma virus integrate their proviruses in the unique DNA region. Finally, in heterologous nonpermissive, but transformable, mouse 3T3 cells, both unique and reiterated host DNA sequences may be associated with the site of integration. It would therefore seem that, if cellular reiterated DNA sequences are involved in transcription regulation, they need not be adjacent to the structural genes they control. Alternatively, the oncornaprovirus may contain information that regulates its own transcription.

ACKNOWLEDGMENTS

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