JOURNAL OF VIROLOGY, Mar. 1989, p. 1107–1115
0022-538X/89/031107-09$02.00/0

Vol. 63, No. 3

Acquisition of New Proviral Copies in Avian Lymphoid Cells Transformed by Reticuloendotheliosis Virus

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Received 3 October 1988/Accepted 30 November 1988

The expression of the v-*rel* oncogene of avian reticuloendotheliosis virus (REV-T) transforms and immortalizes very immature avian lymphoid cells. In REV-T-transformed lymphoid cells which were persistently infected with reticuloendotheliosis-associated virus (REV-A), the REV-T proviral copy number increases after the initial integration event. In 23 independently derived REV-T-transformed cell lines, 15 of the 18 virus-producing cell lines had acquired additional proviruses. The rate at which the newly acquired proviral sequences accumulated differed for various cell lines. In some cell lines, additional REV-T proviral copies could be detected as early as 8 months after the initial integration event. A correlation exists between the number of REV-T proviral sequences and the length of time which a given cell line had been propagated in culture. The integration sites occupied by the newly acquired REV-T proviruses were distinct. In contrast, reticuloendotheliosis-associated virus proviral sequences in these REV-T-transformed virus-producing lymphoid cells did not increase during in vitro culture. Furthermore, the acquisition of additional REV-T proviral sequences did not occur in non-virus-producing cell lines. Two of the newly acquired proviral sequences were molecularly cloned and analyzed by restriction endonuclease mapping. Although the newly acquired REV-T proviruses have not sustained major deletions, the viral sequences and the v-*rel* oncogene display numerous restriction enzyme polymorphisms. The cellular flanking sequences of two newly acquired REV-T proviruses analyzed were unique and shared no homology with flanking sequences of the other REV-T proviruses in these transformed cells. The nucleotide sequence of the virus-cellular DNA junctions of one newly acquired provirus and its cellular sequence prior to proviral integration were defined. A 5-base-pair direct repeat of cellular origin was present on each side of the long terminal repeat, indicating that the mechanism of acquisition of additional REV-T proviral sequences used reverse transcription and integration of new REV-T proviral copies.

The infection of a cell by a retrovirus generally results in the establishment of persistent infection. Initially, a limited number of proviral copies become stably integrated into cellular DNA sequences (12). The status of integrated exogenous proviral copies in cells which have been cultured in vitro over a long time frame, however, has not been described. Hematopoietic cells transformed by the v-*rel* oncogene of avian reticuloendotheliosis virus (REV-T) provide a good system in which to monitor the fate of integrated proviral sequences in long-term persistently infected clones.

REV-T, like most other acutely transforming retroviruses, had deleted some sequences required for virus replication during the acquisition of its oncogene (3, 17). REV-T, therefore, coreplicates with a helper virus, termed reticuloendotheliosis-associated virus (REV-A) (10). Transformation of lymphoid cells by REV-T does not require a helper virus function (14). The expression of the v-*rel* oncogene immortalizes cells and blocks very early avian lymphoid cell differentiation. Hematopoietic cells transformed by the v-*rel* oncogene possess lymphoblastoid morphology, contain low levels of terminal deoxynucleotidyl transferase activity, and weakly express B and T cell surface determinants but generally fail to synthesize immunoglobulin molecules (1, 14, 21). In certain REV-T-transformed lymphoid cells, both the heavy and light immunoglobulin chain genes are in embryonic configuration; therefore, REV-T cells are among the earliest cells transformed by a retrovirus (6).

Some T-cell lymphomas of mice contain newly acquired mouse mammary tumor virus (MMTV) proviral sequences (7, 11, 18). These additional MMTV proviruses have extensive restriction enzyme polymorphisms in their structural genes and a deletion which encompasses part of the glucocorticoid responsive element in the MMTV long terminal repeat (LTR). The acquisition of new endogenous MMTV proviral copies takes place in cells in which virus replication has not been detected. Whether the acquisition of these variant MMTV proviruses provides the cell with a growth advantage or contributes to T-cell lymphomatogenesis is unknown.

In this report, we describe that additional REV-T proviral sequences accumulate after the initial integration event in REV-T-transformed cells persistently infected with REV-A. The newly acquired REV-T proviruses had 5-base-pair (bp) direct repeats of cellular origin flanking the viral LTR, suggesting that the mechanism of acquisition of these new proviruses is through reverse transcription and integration of new REV-T proviral copies.

MATERIALS AND METHODS

Virus and transformed cells. REV-T-transformed lymphoid cells were grown as suspension cultures in RPMI 1640 medium supplemented with 3.3% fetal calf serum and 6.6% calf serum. RECC-UT1 initially was isolated and subsequently cloned from an REV-T-infected bird (8). This cell line had been continuously cultured for 13 years. The RECC-UT1 cells analyzed in this study had been cultured for 1 year [RECC-UT1(1 year)] and stored in liquid nitrogen for 12 years. The RECC-UT1A and RECC-UT1B cells were uncloned progeny of the initial RECC-UT1 isolate which had been separated from the continuously cultured stock at approximately 3 years. RECC-UT1/370 and RECC-UT1/377

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were cultures subcloned in soft agar from RECC-UT1A after RECC-UT1A had been continuously cultured for 8 years.

The procedure for the development of new independently derived REV-T-transformed lymphoid cell lines has been previously described (10). Single-cell suspensions used in the transformation process were prepared from spleens of Hyline SC chickens (Hyline International, Johnston, Iowa). The individual clones isolated from soft agar were screened for virus production by a reverse transcriptase assay (25) and the ability of culture fluid to induce a neoplastic disease in 1-week-old chicks. These new isolates were shown to be independently derived since each contained a unique REV-T (and REV-A) proviral integration site(s).

The REV-A stock used in the rescue of RECC-UTC4-1 was obtained by endpoint dilution from culture fluids of a REV-T-transformed virus-producing cell line. The REV-A stock was amplified in chicken embryo fibroblast cultures. The amplified stock failed to induce a neoplastic disease when infected in chicks, indicating the absence of REV-T contamination.

Construction of probes against different regions of REV-T and REV-A proviral sequences. To prepare a *rel*-specific probe, pIC REV-TΔ*Xba*I (4) was subcloned into pUC18 (27). The complete restriction map of REV-T has been described by Chen and Temin (4). The plasmids containing the 1-kbp sequence between the two *Eco*RI sites were designated pJZ12(*rel*). To prepare a *gag*-specific probe, the *Xba*I fragment between the sites at 1.0 and 2.0 kbp of plasmid pSW253 (3, 26) was subcloned into pUC18. This resulting plasmid was designated pJZ14(*gag*). To prepare a REV-A-specific probe, the *Sma*I fragment between the sites at 2.9 and 3.8 kbp of pSW253 was cloned into pUC18 and the resulting plasmid was designated pJZ15(A.S.). To prepare a probe against the LTR of reticuloendotheliosis virus, an 800-bp *Nru*I fragment to the right of the *rel* gene in pIC REV-TΔ*Xba*I (4) was subcloned into pUC18. This plasmid was designated pJZ27(LTR).

Preparation of high-molecular-weight DNA. Cells (1×10^8 to 3×10^8) were lysed in 20 mM Tris hydrochloride (pH 7.4)–25 mM EDTA–75 mM NaCl–0.5% sodium dodecyl sulfate–100 μg of proteinase K per ml at 37°C for 1 h. The DNA was extracted with phenol-chloroform (1:1) until the interphase was clear. This preparation then was extracted once with chloroform. The DNA was precipitated with two volumes of cold ethanol and then was spooled around a glass rod, dried, and suspended in TE buffer (10 mM Tris hydrochloride [pH 7.6], 0.1 mM EDTA). RNase (1 μg/ml) was added and incubated at 37°C for 1 h. The phenol-chloroform extraction procedure was repeated, and the DNA was suspended in TE buffer. These DNA preparations were from cell lines which had been maintained in culture for different lengths of time as indicated in the text.

Southern transfer. DNA (10 μg) was digested with 50 to 100 U of the specified restriction enzyme in 55 μl. A portion of the digestion mixture (5 μl) was removed and plasmid DNA was added to monitor the completion of the enzyme reaction. Digested DNA fragments were separated by agarose gel electrophoresis in 1× TEA buffer (50× TEA is 2.5 M Tris–1.0 M sodium acetate–0.1 M EDTA [pH 8.05]). The gels were treated in denaturing solution containing 0.5 M NaOH and 1.5 M NaCl for 1.5 h followed by neutralizing solution containing 0.5 M Tris (pH 7.4) and 3 M NaCl for 1.5 h at room temperature. The DNA was transferred onto nitrocellulose paper (23) with 10× SSC (1× SSC is 0.15 M NaCl plus 0.15% sodium citrate [pH 7.0]) for 48 to 72 h. The nitrocellulose paper was baked at 80°C in a vacuum oven for 2 h and then sealed in a plastic bag (Sears Seal-N-Save) for hybridization.

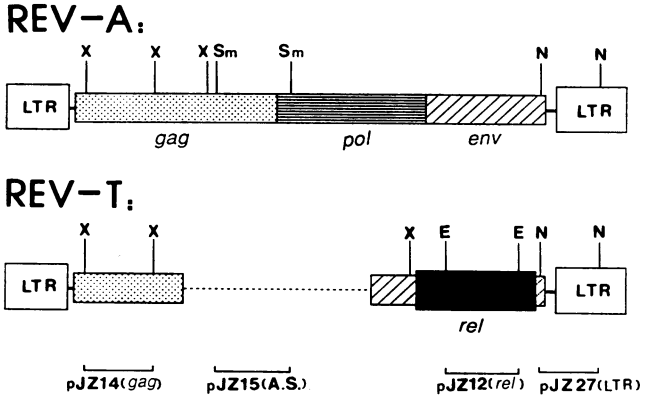

FIG. 1. Molecular probes against different regions of REV-A and REV-T proviruses. The complete restriction maps of REV-A and REV-T were described by Chen and Temin (4). pJZ12 contains the subclone of REV-T between two *Eco*RI sites which was used as a REV-T-specific probe. pJZ15 contains the subclone of REV-A between the *Sma*I sites at 2.9 and 3.8 kbp which was used as a REV-A-specific probe. pJZ14 contains the subclone of REV-A between the *Xba*I sites at 1.0 and 2.0 kbp which was used as a *gag* probe. pJZ27 contains the subclone of REV-T between two *Nru*I sites 3′ to the *rel* sequence which was used as a LTR probe. The abbreviations for the restriction enzymes are as follows: E, *Eco*RI; N, *Nru*I; Sm, *Sma*I; X, *Xba*I.

Hybridization conditions. Southern blots were prehybridized overnight in hybridization buffer containing 50% formamide, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), 2× Denhardt solution (50× Denhardt solution is 5 g of Ficoll, 5 g of polyvinylpyrrolidone, 5 g of bovine albumin fraction V in 500 ml), 0.4% sodium dodecyl sulfate, 3× SSC, 100 μg of yeast tRNA per ml, and 50 μg of heat-denatured salmon sperm DNA per ml at 41°C. Subsequently, excess buffer was removed and replaced by 3×10^7 cpm of heat-denatured probe in 1.5 ml of hybridization buffer, and the mixture was incubated at 41°C for 48 to 72 h. Filters were washed with 1× Denhardt solution–2× SSC at room temperature for 15 min, 2× SSC at room temperature for 45 min, and 0.1% sodium dodecyl sulfate–0.1× SSC at 53°C for 1 to 4 h. Filters were air dried at room temperature for 1 h and exposed to Kodak XAR-2 film in the presence of intensifying screens at −70°C for 1 to 3 days. Filters used for hybridization to more than one probe were placed in 6 ml of hybridization buffer in plastic bags and heated to 65°C for 30 min. The solution was removed, and the procedure was repeated once before filters were rehybridized as described above.

Preparation of molecular clones of virus-cellular DNA junction flanking sequence. To prepare junction fragments containing 3′ virus-cellular DNA flanking sequences, a recombinant DNA library in λgtWES.λB (13) was constructed from a complete *Eco*RI digest of RECC-UT1/370 cell DNA as described by Maniatis et al. (15). The library was screened by hybridization to a nick-translated *rel* probe pJZ12 (Fig. 1). To obtain the cellular sequence prior to proviral integration, a recombinant DNA library was constructed from a complete *Eco*RI digest of MSB-1 cellular DNA. MSB-1 is a avian lymphoid cell line transformed by Marek's disease virus (16). The MSB-1 library was screened by hybridization to nick-translated probes pJZ47 and pJZ48 (see Fig. 6). To prepare junction fragments containing 5′ virus-cellular DNA flanking sequences, the RECC-UT1/370 library was

TABLE 1. Origin of REV-T-transformed lymphoid cells

Cell line	Length of time in culture	Approximate REV-T proviral copy no.	Virus production[a]
RECC-UT1	1 yr	2	+
RECC-UT1B	8 yr	3	+
RECC-UT1B	10 yr	4	+
RECC-UT1A	8 yr	7	+
RECC-UT1A	10 yr	8	+
RECC-UT1A	13 yr	14	+
RECC-UT1/377	8 yr	9	+
RECC-UT1/377	10 yr	11	+
RECC-UT1/370	10 yr	8	+
RECC-UT716	1 mo	2	+
RECC-UT716	11 mo	4	+
RECC-UT716	16 mo	6	+
RECC-UT1W101	1 mo	1	+
RECC-UT1W101	8 mo	3	+
RECC-UTC4-1	6 yr	1	−
RECC-UTC4-1	9 yr	1	−
RECC-UTC4-1R	9 yr	2	+
RECC-UT723	1 mo	1	−
RECC-UT723	16 mo	1	−
RECC-UTCS04	6 mo	2	+
RECC-UTCS04	1 yr	2	+
RECC-UTE11	1 mo	1	+
RECC-UTE11	8 mo	1	+
RECC-UTNH22	1 mo	1	+
RECC-UTNH22	8 mo	5	+
RECC-UT1W11	1 mo	1	+
RECC-UT1W11	8 mo	1	+
RECC-UT1W41	1 mo	1	−
RECC-UT1W41	8 mo	1	−
RECC-UT1W81	1 mo	1	−
RECC-UT1W81	8 mo	1	−
RECC-UT2W21	1 mo	1	+
RECC-UT2W21	8 mo	2	+
RECC-UT2W51	1 mo	1	+
RECC-UT2W51	8 mo	4	+
RECC-UT2W62	1 mo	1	+
RECC-UT2W62	8 mo	2	+
RECC-UT2W72	1 mo	1	+
RECC-UT2W72	8 mo	2	+
RECC-UT4W11	1 mo	1	−
RECC-UT4W11	8 mo	1	−
RECC-UT4W61	1 mo	1	+
RECC-UT4W61	8 mo	2	+
RECC-UT6W11	1 mo	2	+
RECC-UT6W11	8 mo	3	+
RECC-UT6W22	1 mo	1	+
RECC-UT6W22	8 mo	3	+
RECC-UT6W61	1 mo	1	+
RECC-UT6W61	8 mo	2	+
RECC-UT8W71	1 mo	1	+
RECC-UT8W71	8 mo	2	+
RECC-UT8W91	1 mo	1	+
RECC-UT8W91	8 mo	2	+
RECC-UT12W23	1 mo	1	+
RECC-UT12W23	8 mo	3	+

[a] +, Virus producing; −, non-virus producing.

screened by hybridization to nick-translated PJZ14(*gag*) and fragments which represented the cellular sequence prior to proviral integration were cloned from the MSB-1 library. Positive plaques were isolated and screened with the same probes. DNA from each bacteriophage was digested with *Eco*RI and analyzed by the Southern method. The proper fragments were isolated from an agarose gel and cloned into plasmid pUC18 or PACYC184 (2) for restriction endonuclease mapping.

DNA sequence analysis. The dideoxy-chain termination method (19) was used for DNA nucleotide sequence analysis.

RESULTS

Integration pattern of REV-T proviral DNA in transformed lymphoid cell lines. The integration pattern of REV-T proviral sequences was analyzed in a number of REV-T-transformed lymphoid cell lines. The cell lines whose integration patterns were studied are listed in Table 1. These included REV-T-transformed lymphoid cells, as well as their subclones which had been in culture from 1 month to 13 years.

To determine the integration pattern of REV-T proviral DNA, cellular DNAs were digested with the restriction endonuclease *Xba*I and the DNA fragments were separated by agarose gel electrophoresis. The DNA was transferred to nitrocellulose filters and hybridized with the REV-T-specific probe pJZ12(*rel*) (Fig. 1). *Xba*I digests the REV-T genome 5′ to the v-*rel* sequence and in cellular flanking sequences 3′ to the REV-T provirus. The number of REV-T proviral copies was determined by the number of DNA fragments which hybridized to this probe. To distinguish between virus-specific *rel* fragments and the cellular homolog c-*rel*, MSB-1, an avian lymphoid cell line transformed by Marek's disease virus (16), was also analyzed. The results are shown in Fig. 2. There were two DNA fragments in MSB-1 cells (lane 1) which hybridized with the v-*rel* probe. These two fragments represent c-*rel* sequences in the chicken genome. In most REV-T-transformed cell lines analyzed, the number of REV-T proviral copies increased during propagation in culture. In the case of RECC-UT1W101, initially a single REV-T provirus copy was stably integrated (Fig. 2, lane 14). Seven months later, this cell line acquired two additional REV-T proviral copies (Fig. 2, lane 15). In the case of RECC-UT1 after 1 year in culture, a limited number of the REV-T proviral copies were stably integrated (Fig. 2, lane 2). During in vitro cultivation of this cell line, additional REV-T proviral copies were acquired (Fig. 2, lanes 3 to 10). After 13 years in culture, this cell line now contains 14 REV-T proviral copies (Fig. 2, lane 7).

The accumulation of additional REV-T proviral copies in REV-T-transformed cells had been detected in a number of

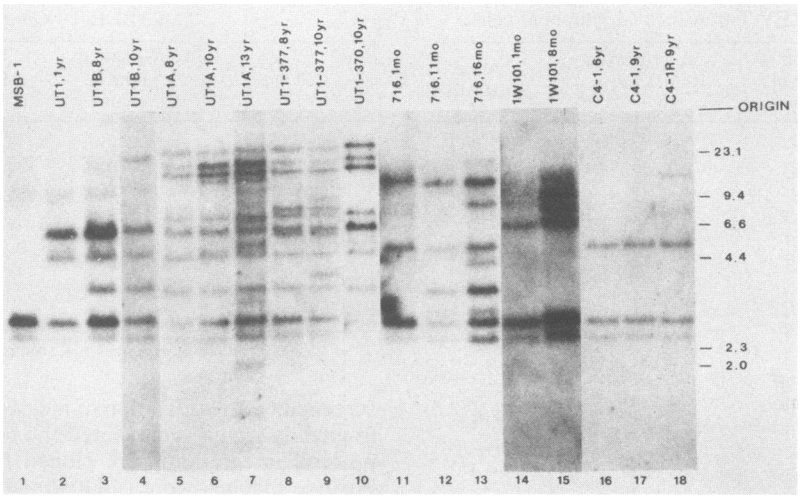

FIG. 2. REV-T proviral sequences in various REV-T-transformed lymphoid cells. DNA (10 μg) from several cell lines was digested with *Xba*I, and the fragments were separated by 0.8% agarose gel electrophoresis and analyzed by the Southern method (23). The fragments homologous to *rel* were detected by using the nick-translated *rel*-specific sequence from pJZ12(*rel*). Molecular weights (10^3) were determined by using *Hin*dIII-digested lambda DNA, as shown on the right. The sources of cellular DNA are indicated on the top of each lane.

cell lines. In 23 independently derived REV-T-transformed cell lines, 15 of the 18 virus-producing cell lines had acquired additional proviral copies after the initial integration event (Table 1).

The integration sites occupied by the newly acquired REV-T proviruses were distinct in all of the cell lines analyzed. The rate at which new REV-T proviral copies accumulated varied for different cell lines. As indicated in Table 1, RECC-UT2W21 acquired one additional REV-T proviral copy after in vitro cultivation for 7 months. RECC-UT1W101 acquired two additional copies in 7 months (Table 1 and Fig. 2, lanes 14 and 15). RECC-UT2W51 acquired three and RECC-UTNH22 acquired four additional REV-T proviral copies after cultivation for 7 months (Table 1). All of these cell lines initially contained a single integrated REV-T provirus. The rate at which new REV-T proviral copies accumulated varied even for different subclones of cell line RECC-UT1. RECC-UT1A acquired 5 additional REV-T proviral copies (lanes 2 and 5) and RECC-UT1B acquired one additional REV-T proviral copy (lanes 2 and 3) during the 7 years these cell lines were cultured. Furthermore, a correlation exists between the number of REV-T proviral sequences and the length of time a given cell line had been propagated in culture. As shown in Fig. 2, the number of REV-T proviral copies increases with increasing time in culture for RECC-UT1 (lanes 2 to 10) and RECC-UT716 (lanes 11 to 13).

The acquisition of REV-T proviral sequences did not occur in the five non-virus-producing cell lines analyzed (Table 1). These cell lines, which did not contain REV-A proviral sequences, were independently derived since each contained distinct REV-T proviral integration sites. RECC-UTC4-1 had been continuously propagated for 9 years and contains a single REV-T proviral copy (Fig. 2, lane 17). When RECC-UTC4-1 was infected with REV-A and then cloned by endpoint dilution (designated RECC-UTC4-1R), a newly acquired proviral copy was detected within 3 years (Fig. 2, lane 18).

The additional *rel* sequences in REV-T-transformed cells are not due to c-*rel* amplification. To determine whether the additional DNA fragments which annealed with the *rel*-specific probe were due to c-*rel* amplification, we hybridized *Eco*RI-digested DNA fragments from two cell lines with *gag*- and LTR-specific probes (Fig. 1). *Eco*RI digests the REV-T proviral genome 5′ to the *gag* gene and in cellular flanking sequences 3′ to the REV-T. The REV-A provirus lacks *Eco*RI sites. MSB-1 cells do not contain DNA fragments which hybridize to the *gag* probe (Fig. 3, lane 1). The cell lines which contained additional fragments which hybridized with the *rel* probe also contained newly acquired fragments which annealed with the *gag* probe (Fig. 3, lanes

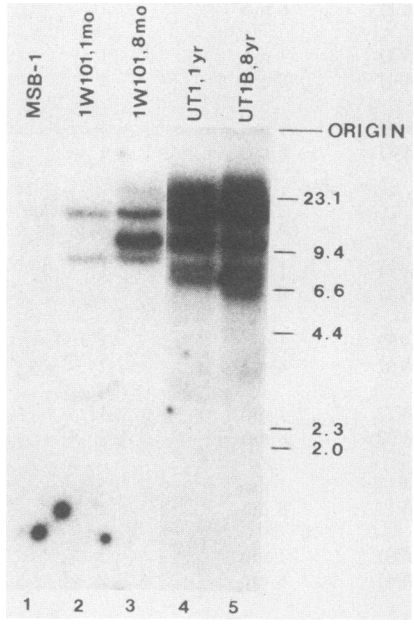

FIG. 3. Hybridization of the *gag*-specific probe with DNA from REV-T-transformed lymphoid cell lines. DNA (10 μg) from several cell lines was digested with *Eco*RI and analyzed by the Southern procedure. Fragments containing *gag* sequences were detected by using the *gag*-specific probe from pJZ14(*gag*). Molecular weights (10^3) were determined by using *Hin*dIII-digested lambda DNA, as shown on the right. The sources of cellular DNA are indicated on the top of each lane.

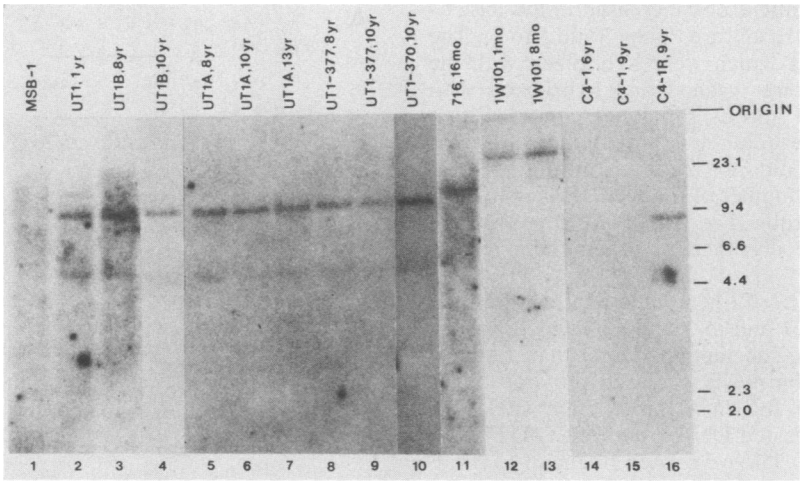

FIG. 4. Detection of REV-A-specific sequences in REV-T-transformed lymphoid cell lines. The filters used for Fig. 2 were washed as described in Materials and Methods and hybridized with the REV-A-specific sequences purified from pJZ15(A.S.). Molecular weights (10^3) are shown on the right.

2, 3 and 4, 5). In addition, the fragments obtained from RECC-UT1 which hybridized to the *rel*-specific probe also annealed to the LTR probe (data not shown). Therefore, the additional *rel*-related sequences detected in these REV-T-transformed lymphoid cells were not due to c-*rel* amplification.

Integration pattern of REV-A in REV-T-transformed lymphoid cell line. Comparison of the restriction maps of REV-A and REV-T proviral DNA (4) indicated that the *Sma*I fragment from 2.9 to 3.8 kbp was REV-A specific. To construct a REV-A-specific clone, we subcloned this *Sma*I fragment from pSW253, a plasmid containing a biologically functional REV-A genome (3, 26), into pUC18. The resulting construct was designated pJZ15(A.S.) (Fig. 1). This *Sma*I fragment hybridized with REV-A sequences but failed to hybridize with REV-T (data not shown).

To determine whether REV-T-transformed lymphoid cells also acquired REV-A proviral copies during passage in vitro, the same nitrocellulose filters which were hybridized with the REV-T-specific probe were washed and hybridized with the REV-A-specific probe pJZ15(A.S.) (Fig. 4). *Xba*I digests the REV-A proviral genome 5′ to the *Sma*I fragment which we subcloned as a REV-A-specific probe and in flanking cellular sequences 3′ to the REV-A provirus (Fig. 1). DNA from MSB-1 lacked fragments which hybridized to the REV-A-specific probe (Fig. 4, lane 1). In the non-virus-producing cell lines (e.g., RECC-UTC4-1), REV-A-specific sequences were not detected (lanes 14 and 15). In all virus-producing cell lines, there were one to two copies of REV-A integrated at different cellular sites. RECC-UT1 and its subclones contained two REV-A proviral sequences (lanes 2 to 10), and therefore REV-A proviral copies did not increase during in vitro passage of these lymphoid cells. Similar results were observed in cell lines RECC-UT716 (lane 11) and RECC-UT1W101 (lanes 12 and 13) and other cell lines (data not shown). The results were the same when *Eco*RI was used (data not shown). In addition, since the REV-A-specific probe hybridized principally to a single *Xba*I fragment obtained from these cell lines, the increase in the number of fragments which hybridized to the *rel*-specific probe (Fig. 2) cannot be explained by incomplete digestion or other endonuclease activity.

Cloning of the cellular flanking sequence adjacent to a newly acquired REV-T provirus. DNA from the RECC-UT1 cell line was also digested with the restriction enzyme *Eco*RI. The *rel* probe (pJZ12/*rel*) used in these studies contained the sequences between the two *Eco*RI sites of the v-*rel* gene (Fig. 1). MSB-1 cells contained two *Eco*RI fragments (9.0 and 17.0 kbp in length) which were recognized by this v-*rel*-specific probe (Fig. 5, lane 1). These represent the two internal fragments of the c-*rel* proto-oncogene (5). Irrespective of the number of integrated REV-T proviral copies, one would anticipate that all REV-T-transformed cell lines would exhibit three *Eco*RI fragments (2 c-*rel* fragments and a 1.0-kbp middle segment of the v-*rel* gene). In all cell lines transformed by REV-T (for example, RECC-UTC4-1 [Fig. 5, lane 2]), three DNA *Eco*RI fragments hybridized with the v-*rel* probe. However, *Eco*RI-digested DNA from RECC-UT1 and its subclones (lanes 3 to 10) were found to contain a number of additional fragments that hybridized with the middle v-*rel* probe. The number of *Eco*RI fragments which

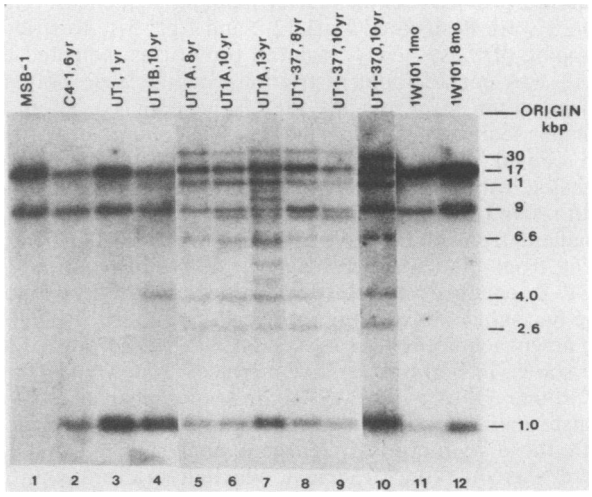

FIG. 5. REV-T proviral sequences in REV-T-transformed lymphoid cells. DNAs from several different REV-T-transformed cell lines were digested with *Eco*RI and hybridized with the *rel*-specific fragments identified as described in the legend to Fig. 2. Molecular weights (10^3) are shown on the right.

hybridized to the *rel*-specific probe increased in the RECC-UT1 cell line during in vitro culture (lanes 3 and 5 to 7). The same nitrocellulose filters which were hybridized with the REV-T-specific probe were washed and hybridized with pJZ15(A.S.). RECC-UT1 and its subclones contained two REV-A proviral sequences (data not shown), indicating that REV-A proviral copies did not increase during in vitro passage. The increase in number of fragments which hybridized to the *rel*-specific probe (Fig. 5) cannot, therefore, be explained by incomplete digestion or other endonuclease activity.

Since several of the *Eco*RI-generated DNA fragments obtained from RECC-UT1 and its subclones which hybridized to the *rel* probe were substantially larger than 1 kbp, it appeared that some of the newly acquired proviral copies had lost one or both of the *Eco*RI restriction sites. We hybridized the *Eco*RI-digested DNA from RECC-UT1 with the LTR and *gag* probes. The *rel*-related fragments hybridized with the LTR-specific probe but failed to hybridize with the *gag*-specific probe (data not shown). Therefore, the newly acquired *Eco*RI fragments observed in the digestion of RECC-UT1 were derived from REV-T proviruses which had lost the 3′ *Eco*RI site. In RECC-UT1 cultured for 1 year (Fig. 5, lane 3), there were no fragments without the 3′ *Eco*RI site in the v-*rel* sequence. Therefore, those *rel*-containing fragments which lost their 3′ *Eco*RI sites were acquired after the initial integration event. Not all of the newly acquired REV-T proviral copies displayed this *Eco*RI polymorphism. The newly acquired REV-T provirus in RECC-UT1W101 (Fig. 5, lane 12) did not lose this *Eco*RI site.

To define the mechanism by which new REV-T proviruses accumulate in persistently infected cells, two newly acquired proviruses were molecularly cloned. We cloned two proviruses which had lost the 3′ *Eco*RI sites, since these proviruses had been acquired after the initial integration event. Cellular DNA from cell line RECC-UT1/370 was digested completely with *Eco*RI and ligated into *Eco*RI-digested λgtWES.λB DNA. After packaging, *Escherichia coli* K803 was infected and screened by hybridization with a *rel*-specific probe [pJZ12(*rel*)]. From five positive clones, two clones containing a 2.6- or a 6.6-kbp fragment were obtained. These two fragments represent the 3′ ends of two newly acquired provirus-cellular DNA flanking sequences. These clones were designated Lgt1-12-2 and Lgt1-5-1. Restriction mapping of these newly acquired proviruses indicated that there was considerable restriction endonuclease polymorphism within v-*rel* and the LTR (Fig. 6A). To obtain the cellular sequence flanking the newly acquired proviruses, the cloned virus-cellular DNA flanking sequences were digested with several restriction enzymes and hybridized with *rel* and LTR probes. The sequence in the *Sma*I-*Eco*RI fragment from clone Lgt1-12-2 and the *Hin*dIII-*Pst*I fragment from clone Lgt1-5-1 failed to hybridize with viral REV-T sequences and were subcloned and used as probes. The two subclones containing the cellular sequences flanking the newly acquired proviruses, designated pJZ47 and pJZ48, are shown in Fig. 6A.

Cellular DNA from MSB-1 and a number of REV-T-transformed cells were digested with *Eco*RI and hybridized with these two probes. Southern analysis indicated that there was only one fragment which hybridized with the probes in MSB-1, RECC-UTC4-1, and RECC-UT1B (Fig. 7, lanes 1 to 3), indicating that these fragments represent a unique sequence in chicken chromosomal DNA. In addition to the shared fragment, RECC-UT1A and its subclone RECC-UT1/370 (Fig. 7, lanes 4 and 5) contained an additional fragment which hybridized with pJZ47 and pJZ48 and also hybridized with the *rel* probe (Fig. 5, lanes 6 and 10). The two fragments that hybridized to pJZ47 (Fig. 7A) represent two alleles of the same locus. The two fragments that hybridized to pJZ48 (Fig. 7B) represent alleles of a different locus. One of these alleles in each case did not have an integrated REV-T provirus, since the chicken chromosome is diploid. Southern analysis also demonstrated that there was no significant homology between the cellular DNA flanking sequences of the other newly acquired REV-T proviral copies.

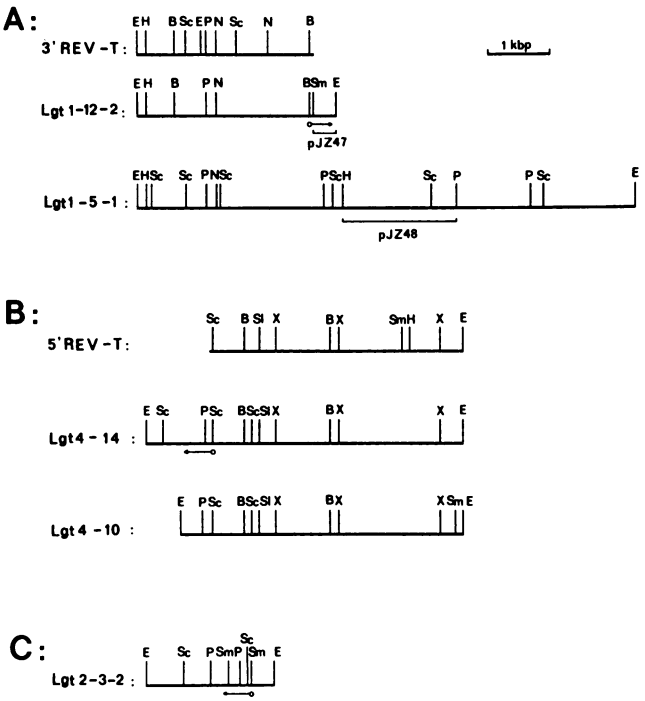

FIG. 6. Restriction map analyses of the virus-cellular DNA flanking sequence of two newly acquired REV-T provirus DNAs. (A) Restriction map of 3′ REV-T and molecularly cloned 3′ ends of virus-cellular DNA sequences of two newly acquired proviruses. (B) Restriction map of 5′ REV-T and 5′ ends of virus-cellular DNA sequences of the two newly required proviruses. (C) Restriction map of the cellular sequence prior to proviral integration. The sequencing direction is shown by an arrow under the restriction map. The abbreviations for the restriction enzymes are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nru*I; P, *Pst*I; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; X, *Xba*I.

The newly acquired REV-T proviruses have 5-bp direct repeats of cellular DNA. The most likely mechanism for acquisition of additional REV-T proviruses in REV-T-transformed cells is reverse transcription and integration of the new REV-T copies. To test this hypothesis, the virus-cellular DNA flanking both sides of the newly acquired provirus were defined. Lgt1-12-2 and Lgt1-5-1 lambda DNA contained the 3′ junction of two newly acquired proviruses. Since the cellular flanking sequences were unique, we cloned the cellular sequences prior to proviral integration and used them as probes to clone the 5′ virus-cellular DNA flanking sequences. Cellular DNA from MSB-1 was *Eco*RI digested and ligated into *Eco*RI-digested λgtWES.λB, and bacteria were infected with these phages. The library was screened by hybridization with pJZ47 and pJZ48 probes. Two fragments (2.2 and 5.3 kbp) representing the sequences prior to integration of the newly acquired REV-T provirus in cellular

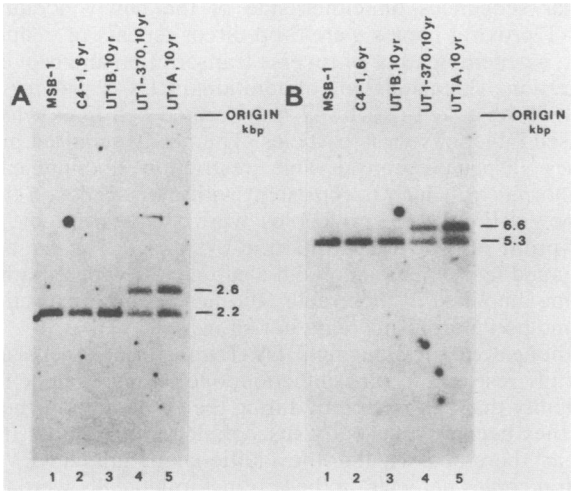

FIG. 7. The cellular sequences surrounding the newly acquired REV-T proviruses are unique. Cellular DNA was digested with *Eco*RI and hybridized with the cellular DNA flanking sequences pJZ47 and pJZ48 (Fig. 6A). (A) Result of a Southern analysis with the probe pJZ47. (B) Result of a Southern analysis with the probe pJZ48. The cell lines examined are listed on the top of each lane. Molecular weights (10^3) are shown on the right.

DNA of RECC-UT1/370 were obtained and the 2.2-kbp fragment was designated Lgt2-3-2. Using the 2.2- and 5.3-kbp fragments and *gag* (pJZ14) as probes, the library of RECC-UT1/370 was screened and the fragments (5.0 and 4.5 kbp) were cloned. These two fragments represent the two 5′ REV-T proviral DNA-cellular-DNA flanking sequences, respectively (designated Lgt4-14 and Lgt4-10). The two fragments were restriction endonuclease mapped. As in the 3′ ends of the REV-T proviruses, the restriction mapping displayed considerable restriction endonuclease polymorphism at the 5′ ends (Fig. 6B).

The junctions of cellular and viral flanking sequence were cloned into M13mp18 and M13mp19 and sequenced by using the dideoxy-chain termination method (19). The sequencing direction is shown in Fig. 6, and the nucleotide sequence is shown in Fig. 8. There were 5-bp direct repeats (TACCC) on each side of the LTR, and the 5 bp corresponded to the cellular sequence prior to proviral integration. These results indicate that acquisition of new REV-T proviral sequences in the transformed lymphoid cells uses the reverse transcriptase of REV-A.

DISCUSSION

In lymphoid cells infected and transformed by REV-T, a limited number of proviral copies initially become stably integrated (12). Although acquisition of additional endogenous retroviral sequences has been reported in murine thymomas (7, 11, 18), the acquisition of additional proviral copies of exogenous retroviruses in persistently infected cells has not been described. In avian lymphoid cells transformed by REV-T and propagated in culture, the number of integrated REV-T proviral copies increases. Cell lines in which the REV-T provirus copy number increased were those which contained REV-A sequences and produced infectious virus. This has been demonstrated for 15 of the 18 independently derived virus-producing cell lines analyzed. The newly acquired REV-T proviral copies were integrated into different cellular sequences suggesting random integration. By contrast, REV-A proviral sequences in these REV-T-transformed lymphoid cells did not increase during in vitro culture.

Southern analyses using two different restriction endonucleases indicated that REV-T provirus acquisition occurred

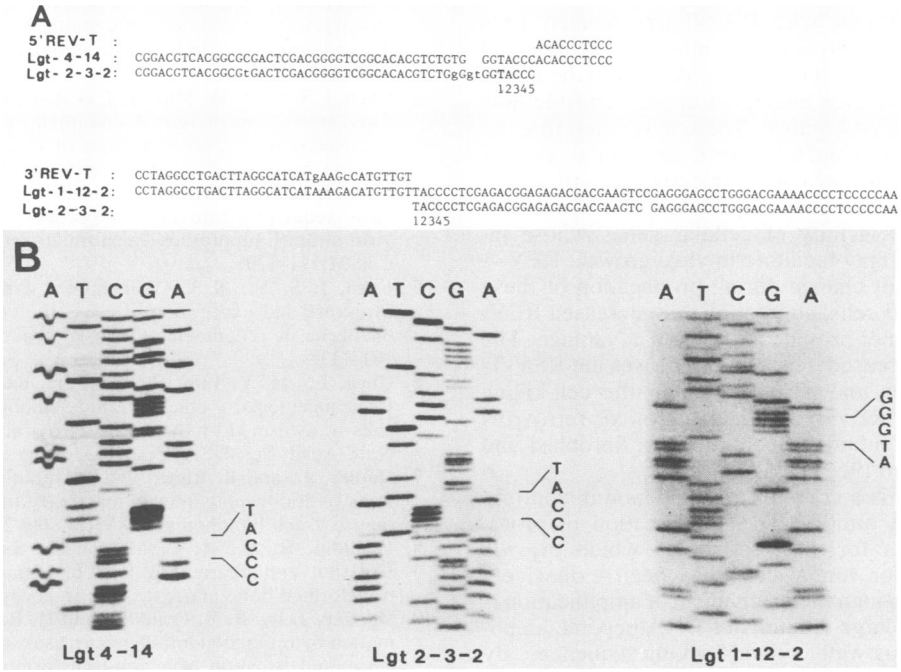

FIG. 8. The nucleotide sequence of the virus-cellular DNA flanking sequences of a newly acquired REV-T provirus. (A) Capital letters indicate the nucleotides which are the same in the virus-cellular DNA flanking sequences (Lgt4-14 and Lgt1-12-2) which correspond to the REV-T sequence (22) and the cellular sequence prior to proviral integration (Lgt2-3-2). The 5-bp direct repeat is identified by numbers. (B) Portion of the sequencing gel which identifies the 5-bp direct repeats. The 5-bp repeat (TACCC) is indicated on the right of each gel.

in these independently derived cell lines. Those filters which were used in hybridization experiments with a *rel*-specific probe were washed and rehybridized with a REV-A-specific probe. The REV-A-specific probe hybridized to two *Xba*I fragments obtained from RECC-UT1 and its subclones. *Eco*RI digestion showed a similar result. The increase in number of fragments which hybridized to the *rel*-specific probe, therefore, cannot be explained by incomplete digestion or other endonuclease activity. Furthermore, in a given cell line the size of the REV-A and REV-T provirus-cellular DNA junction fragments were conserved in both *Xba*I and *Eco*RI digestions, providing evidence that the acquisition of additional REV-T proviral copies occurred in a cell line derived from a single origin.

The addition of REV-T proviral sequences was detected in a very large population of cells. Since the newly acquired REV-T proviruses occupy different integration sites, the acquisition of REV-T proviral DNA would not have been detected in this cell population unless the additional REV-T copies provide the cell with a growth advantage. The failure to detect an increase in REV-A proviral sequences in these REV-T-transformed lymphoid cells is also consistent with the suggestion that the additional REV-T proviruses provide a selective growth advantage to the cells. Why REV-T-transformed lymphoid cells which contain additional REV-T proviral copies were selected during in vitro cultivation is unclear. There are at least two possible explanations why cells containing additional REV-T proviruses outgrow those cells in which the acquisition has not occurred. First, cells containing increased REV-T copies may express more $pp59^{v\text{-}rel}$ and provide the cells with a selective growth advantage. RECC-UTC4-1R, which contains two REV-T proviruses, contains approximately twofold more $pp59^{v\text{-}rel}$ than the cell line RECC-UTC4-1 which contains a single copy (data not shown). In different cell lines, however, the level of $pp59^{v\text{-}rel}$ did not invariably correlate with REV-T proviral copy numbers. Increased levels of $pp59^{v\text{-}rel}$ alone, therefore, cannot explain why REV-T-transformed cells with increased REV-T proviral copy numbers are selected during in vitro propagation. The v-*rel* sequences in the newly acquired REV-T proviruses examined display considerable restriction enzyme polymorphism. There may, therefore, be qualitative differences in $pp59^{v\text{-}rel}$ from some new proviral copies of REV-T that also provide a selective growth advantage. Alternatively, these newly acquired REV-T proviruses may integrate in the vicinity of cellular genes whose increased transcription may facilitate in vitro growth. REV-A proviral copy does not change during propagation of these transformed lymphoid cells, suggesting that increased REV-A proviral copies do not provide a selective advantage. The failure to detect increased REV-A proviruses in REV-T-transformed cell lines may also be due to the cell-killing properties of REV-A. REV-A is a highly cytotoxic retrovirus and induces massive rounds of cell killing in fibroblast and lymphoid cells in vitro (9, 24).

DNA amplification is a common phenomenon in continuous cell lines and in tumors. The amplification of proto-oncogenes and genes for drug resistance which provide growth advantages for tumor cells has been extensively characterized (20). Though the mechanism of amplification is not well understood, large amounts of DNA become amplified, including genes as well as their flanking sequences. By contrast, the acquisition of additional REV-T proviral sequences in transformed avian lymphoid cells described here most likely involved reverse transcription of viral RNA and the subsequent integration of a new copy of viral DNA. The cellular sequences on either side of the newly acquired REV-T proviral copies were 5-bp direct repeats of cellular DNA, a general feature of reverse transcription and proviral integration. The acquisition of additional REV-T proviruses was only observed in REV-T-transformed cell lines which released infectious virus particles. The newly acquired proviruses displayed considerable restriction endonuclease polymorphism, which is consistent with the suggestion that the new REV-T proviral copies were synthesized by an error-prone reverse transcriptase. c-*src*, c-*myc*, 3′ c-*rel*, and heavy and light immunoglobulin chain genes, which did not become amplified in these cells, did not contain restriction enzyme polymorphisms (data not shown).

Although virus-producing REV-T-transformed cells are generally resistant to superinfection, one cannot exclude the possibility that at some point during the cultivation of these cells they become transiently susceptible to reinfection. It is unclear, therefore, whether the additional integrated REV-T proviral copies detected in these transformed cells were the result of reinfection of the cells with exogenous virus or resulted from intracellular reverse transcription and subsequent virus-DNA integration.

ACKNOWLEDGMENTS

We thank James Walker and Jaquelin Dudley for their helpful discussion and comments on the manuscripts, Howard M. Temin for providing us plasmids containing REV-A and REV-T proviruses, and R. L. Witter for sending us the RECC-UT1 cells, which had been in freezer storage for years.

This research was supported by Public Health Service grants CA 33192 and CA 26169 from the National Cancer Institute.

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