Spontaneous Variation and Synthesis in the U3 Region of the Long Terminal Repeat of an Avian Retrovirus

KUNITADA SHIMOTOHNO† AND HOWARD M. TEMIN*

McArdle Laboratory, University of Wisconsin, Madison, Wisconsin 53706

Received 29 June 1981/Accepted 28 August 1981

Retrovirus DNA contains a long terminal repeat (LTR) which has the structure U3-R-U5 (13, 22). U3 and U5 are unique sequences from the 3' and 5' ends of viral RNA, respectively, and R is a short terminal repeat at the ends of viral RNA (reviewed in 5, 28). It has been proposed that only the 3' LTR in proviral DNA is used as a template for synthesis of the U3 region in viral RNA and that, in synthesis of proviral DNA, the U3 region of viral RNA is copied twice to give rise to the U3 region of both LTRs (5, 28).

We have been studying the structure of proviruses of spleen necrosis virus (SNV), an avian retrovirus. In the course of sequencing virus-cell junctions of several SNV proviruses (24), we noted a variation in the size of the 5' LTR. Here we localize that variation to the U3 region of the LTR and show that the U3 sequences of both LTRs are the same in any one provirus and that both are derived from the 3' LTR of parent virus DNA.

MATERIALS AND METHODS

Proviruses were cloned by phage containing proviruses from chicken cells infected with SNV. Proviruses were described previously (18).

Subcloning of SNV provirus DNA. SNV provirus DNAs were digested with SalI and EcoRI. The DNA fragments were inserted between the SalI and EcoRI sites of pBR322 DNA as described previously (23).

DNA sequencing. DNA sequencing was carried out by the method of Maxam and Gilbert (15). The strategy of sequencing part of the 5' LTR region of these cloned DNAs was as follows (see maps in Fig. 1 and 3). Subcloned DNA which contained the 5' LTR sequence was digested with SacI and, after removal of phosphate residues, labeled at its 3' ends with T4 polynucleotide kinase. After the DNA was digested with BamHI, the 0.5-kilobase pair (kbp) fragment which contained the LTR sequence was isolated and sequenced. Another portion of 5' subcloned DNA was digested with AvaI and SacI. The 0.4-kbp DNA fragment which contained most of the U3 region of the LTR was isolated and was digested with TaqI. The 5' ends were labeled with polynucleotide kinase, and the DNA was digested with Hhal (clones 60 and 14-44) or HaeIII (clone 70). The larger DNA fragment was isolated and sequenced.

Restriction endonuclease cleavage. Restriction endonucleases were purchased from New England Biolabs, Inc. DNA was digested under the conditions recommended by the supplier. After incubation, 80% formaldehyde containing 0.01% bromophenol blue and xylene cyanol was added for agarose-acrylamide composite gels, and 30% sucrose-0.1% sodium dodecyl sulfate containing 0.01% bromophenol blue was added for agarose gels.

Agarose-acrylamide composite gels. The method of Floyd et al. (9) was used for the preparation of 7% agarose and 6 or 8% acrylamide composite gels. Gels were made with N, N'-diallyltartardiamide instead of bisacrylamide so that DNA fragments could be transferred to nitrocellulose paper after electrophoresis. Transfer of DNA fragments from gels to nitrocellulose paper was carried out by the method of Reiser et al. (21) with some modifications. Briefly, the gels were soaked in 5% perchloric acid at 37°C for 20 min with gentle shaking, treated with 0.5 N NaOH for 20 min, and neutralized with 1 M Tris-hydrochloride (pH 7.0)–0.6 M NaCl. The gels were placed on Whatman filter paper as in the Southern procedure, and DNA fragments were blotted onto nitrocellulose paper instead of diazobenzoyloxymethyl-paper.

Transfection of chicken cells with DNA. Chicken cells were transfected with recombinant SNV DNA by the calcium phosphate coprecipitation technique in the presence of salmon sperm DNA as carrier (2). Four or five days after transfection, cytopathic effects were observed and virus was harvested.

Extraction of unintegrated viral DNA from virus-infected chicken cells. Cells were harvested 3 days after infection with virus harvested after transfection. Unintegrated viral DNA was separated from chromosomal DNA by the Hirt procedure (12). The Hirt supernatant fraction was digested with proteinase K (final concentration, 50 μg/ml) at 37°C for 2 h followed by RNase A

† Present address: National Institute of Genetics, Mishima 411, Japan.
from P-L Biochemicals, being dissolved in buffer, the DNA was digested with restriction endonucleases.

Materials. Agarose, acrylamide, N,N'-diallyltartardiamide, and bisacrylamide were purchased from Bio-Rad Laboratories. Alkaline phosphatase was from Worthington Diagnostics. Polynucleotide kinase was from P-L Biochemicals, Inc. Nitrocellulose paper was from Schleicher & Schuell Co.

RESULTS

Identification of variability in the 5' LTR by restriction enzyme mapping. To look for alterations in the size of the 5' LTR, DNAs of 5' subclones of provirus clones 60, 70, and 14-44 were digested with restriction endonucleases known to cleave once in the LTR and were analyzed in a 7% agarose-6% acrylamide composite gel (Fig. 1). The right three lanes of Fig. 1 contain the region of the gel with the SacI fragment containing the LTR from each of the clones (data not shown). The size of this DNA fragment containing the LTR sequence differed among the three cloned proviruses.

To localize this size difference, the same DNAs were digested with SacI plus BamHI (middle three lanes in Fig. 1). The SacI fragment which contained the LTR sequence was cut once by digestion with BamHI, yielding fragments of about 500 and 220 base pairs (bp). The larger DNA fragment had an altered size in each clone, whereas the smaller DNA fragment had the same size in each of the three cloned DNAs.

After digestion with SacI and AvaI (left three lanes in Fig. 1), the DNA fragments which migrated between 400 and 340 bp contained the LTR sequence. Again, the faster migrating DNA, approximately 350 bp, was the same size in all the cloned DNAs, but the slower migrating DNAs differed in size. The differences in mobility of these DNA fragments localized the alteration in size to the U3 region left of the AvaI cleavage site.

To look for alterations in the LTRs of other cloned proviruses, subclones containing the 5'

---

FIG. 1. Cleavage pattern of 5' subcloned DNA of clones 60, 14-44, and 70. pBR322 DNA digested with HinfI produced the size markers in this and succeeding figures. The procedure by which 5' subcloned DNAs were digested with the indicated enzymes and analyzed in gels is described in Materials and Methods. The drawing below the picture is a part of the 5' subcloned DNA: the box indicates 5' LTR; the sawtooth line, cellular DNA; and the solid line, viral DNA; the numbers indicate sizes of fragments in bp in clone 14-44. The numbers at the top indicate the proviral DNA clone. (The largest fragment from the SacI digestion of these DNAs, more than 5 kbp, the shortest fragment of subclone 60, 250 bp, and the shorter fragment from the SacI + BamHI digestion, 220 bp, are not shown.) The fastest migrating band from the SacI-AvaI digestion, approximately 350 bp, migrated anomalously. It contained 371 bp.
LTR from four other clones were also analyzed after digestion with SacI and AvaI (Fig. 2). The DNA fragments containing the LTR region migrated between marker DNAs of 506 and 344 bp, except for clone 32. In clone 32, the SacI cleavage site at 0.75 kbp was missing, leading to the loss of the 350-bp fragment. There were three sizes for the left SacI-AvaI fragment containing the U3 sequences: clones 4 and 44 were the same size as clone 60, clones 32 and 36 were the same size as clone 14-44, and clone 70 was smaller than the others. The alterations in size were about 20 bp.

To localize further the alterations in LTR sequence, the 5' subclones were digested with SacI, and the DNA fragments which contain most of the LTR sequence were isolated. These fragments were then cleaved with AvaI and TaqI (Fig. 3A). Six DNA bands were found. Five DNA fragments, 211, 186, 127, 47, and 11 bp, were the same size in all clones (the latter two are not shown). The nucleotide regions to which these DNA fragments correspond is indicated below the figure. The DNA band which migrated differently in the three clones was 196 bp in clone 60, 170 bp in clone 14-44, and 149 bp in clone 70 and corresponded to the leftmost nucleotides.

Identification of variability in the 3' LTR by restriction enzyme mapping. A strategy similar to that detailed above was used to analyze variability in the 3' LTRs of these proviruses. Upon digestion with SacI and AvaI, multiple bands were resolved by 7% agarose–6% acrylamide gel electrophoresis (Fig. 4A). The bands corresponding to the 3' LTR were identified by hybridization to a 32P-labeled probe made from the 750-bp SacI fragment in the LTR region of the 5' subclone of clone 14-44 (see maps in Fig. 3 and reference 18) (Fig. 4B). In clones 60 and 4, the DNA fragment which migrated with the 400-bp marker hybridized to the probe (indicated by "a"). This fragment contained most of the U3 sequences. In clone 44, in addition to the band which had the same size as that of clones 60 and 4, a faster migrating band also hybridized with the probe. This faster migrating DNA fragment contained part of the 3' LTR sequence and some cellular sequences adjacent to the 3' end of the provirus (data not shown). In clone 14-44, a major band was seen at a position just below the band of clone 60 (indicated by "b" on Fig. 4). This band was also present in clones 36 and 32. In clone 36, an additional band was seen below the common band. This faster migrating band contained the R and U5 region of the 3' LTR in addition to some cellular sequences adjacent to the viral DNA (data not shown). (The larger DNA bands which hybridized resulted from partial digestion of the DNA.) In clone 70, the band containing the U3 region of the 3' LTR migrated faster than the corresponding band of clone 14-44 (indicated by "c"). These results show that the LTRs of the 3' region of the provirus also have U3 regions of different sizes in different clones. Furthermore, proviruses which have a short 5' LTR have a short 3' LTR and proviruses which have a long 5' LTR have a long 3' LTR.

To localize further the differences between clones, the SacI-BamHI DNA fragment which contained most of the 3' LTR was digested with AvaI and TaqI and was analyzed. Four DNA bands were present in every case (Fig. 3B). Three DNA fragments, 211, 97, and 47 bp, were
the same size in each clone. They corresponded in clone 14-44 DNA to nt 182 to nt 392 (211 bp), nt 440 to nt 536 (97 bp), and nt 393 to 439 (47 bp). Therefore, the DNA band which was different in size in each clone corresponded to the DNA fragment nt 12 to 181 (170 bp) in clone 14-44. This result indicates that the differences in the 3' LTR are present in the same region and have the same relative size as those in the 5' LTR.

DNA sequence of U3 region in 5' LTR of clones 14-44, 60, and 70. Restriction enzyme analysis has localized the variation in the LTR to the U3 region between nt 12 and 181. Therefore, this part of the U3 region in the 5' LTR of one cloned DNA of each size class was sequenced (Fig. 5). Clones 60 and 70 have an additional 26 nucleotides between nt 150 and 151 relative to clone 14-44. This additional sequence had the same sequence as nt 151 through 176 except for one base change, T to C at nt 172. In addition, clone 70 had a 46-nucleotide deletion between nt 77 through 124 relative to clone 14-44. All other sequences in this region were the same in these three cloned DNAs. The sequences agree with the sizes predicted from restriction enzyme cleavage experiments.

Construction of recombinant SNV proviruses carrying different-sized LTRs at the two ends and transfection of chicken fibroblast cells with these DNAs. To test whether the 5' and 3' LTRs have the same U3 sequence as a result of the use of only one proviral U3 sequence in replication, we made recombinant proviruses with the 5' LTR from one provirus and the 3' LTR from another provirus with LTRs of a different size. The 5' subcloned DNA of clone 14-44 and the 3' subcloned DNA of clone 60 were digested with SalI and ligated to each other. In a similar fashion, the reciprocal recombinant was constructed. These DNAs and the parental DNAs were used in transfection of chicken fibroblast cells, and unintegrated viral DNAs were obtained as described in Materials and Methods. The DNAs were digested with SacI and AvaI, were resolved in a 7% agarose-6% acrylamide gel, and were transferred to nitrocellulose paper. To identify the size of the LTRs, the paper was hybridized with the 32P-labeled SacI DNA fragment of 5' subclone 14-44, which carries most of the 5' LTR and some viral DNA adjacent to the 5' LTR.

As can be seen in the right two lanes of Fig. 6,
the parental DNAs differed only in the largest SacI-AvaI fragment which contained most of the U3 region of the LTR. This largest DNA fragment from clone 14-44, 381 bp, was shorter by 26 bp than the corresponding fragment of clone 60, 407 bp. In the virus from the recombinants which had the 5' LTR of clone 14-44 and the 3' LTR of clone 60, the largest DNA fragment was 407 bp, the same size as that from clone 60. In the reciprocal recombinant, which had the 5' LTR of clone 60 and the 3' LTR of clone 14-44, the largest DNA fragment was 381 bp, the same size as that from clone 14-44. This result indicates that the U3 sequence of the 5' LTR of unintegrated viral DNA derives only from the 3' LTR of proviral DNA and, therefore, that the U3 regions of both LTRs derive from the same sequences.

**DISCUSSION**

**Synthesis of the LTR.** We found variability in the size of the U3 region of LTRs of different SNV proviruses. In all cases both LTRs were the same size in any one provirus (summarized in Fig. 7).

This similarity of the U3 regions of both LTRs of any one provirus clone indicates that both must have come from a common precursor; that is, the variation did not arise during cloning. To determine whether the 5' or 3' U3 sequences, or both, of the LTR are ancestral to progeny virus, we constructed recombinant proviral clones with different-sized LTRs at each end and used these recombinants in transfection experiments. The data shown in Fig. 6 indicate that only the U3 region of the 3' LTR is ancestral to the 5' and 3' U3 regions of progeny virus.

After proviral DNA enters chicken cells during transfection of chicken cells by viral DNA in the presence of calcium phosphate, it is thought that viral RNA is transcribed (6). It has been shown that the 3' LTR sequence of avian sarcoma virus has promoter activity in vitro (29). Since the 5' LTR has the same structure as the 3' LTR, it is very likely that the 5' LTR also acts as a promoter for transcription of viral RNA. In SNV, RNA synthesis might start at the 5' end of R in the 5' LTR, using a promoter in the U3 region. Thus, the viral RNA no longer has the U3 region of the 5' LTR of the parent. Upon
reverse transcription, the U3 region of the 5′ LTR would be reconstituted from the U3 sequence of viral RNA by “jumping synthesis” (4, 10, 26, 28).

The U3 region in the 5′ LTR thus apparently has at least two different functions: to act as a promoter for transcription of viral RNA and to participate in integration into host DNA. For both functions, the size of the U3 sequence appears not to be critical. Thus, some proviruses have longer U3 sequences and others have shorter sequences. These variations in the U3 region were observed about 200 bp upstream from the postulated control signal for RNA synthesis, TATATAA at nt 366 to 372 in clone 14-44. Therefore, it appears that a 26- or 46-bp duplication/deletion 250 bp upstream from the 5′ end of viral RNA does not prevent viral RNA synthesis. By a similar argument, it appears that 26- or 46-bp deletions or insertions about 80 bp inside the 5′ ends of the U3 region do not prevent integration of viral DNA into cellular DNA or synthesis of + strand DNA. However, no provirus was found with both deletions.

A possible analogy is that simian virus 40 has a 72-bp duplication in its early region at an analogous location with respect to the 5′ end of viral RNA. The duplication can be deleted and expression will still occur. However, deletion into the remaining copy eliminates expression (3; J. Mertz, personal communication).

Mooney murine sarcoma virus also has a 72-bp duplication at an analogous location (7, 20), whereas Mooney murine leukemia virus does not (27). The two duplications in SNV, 26 and 46 bp, add up to 72 bp.

**Mechanisms of variation in proviral DNA.** Sequencing of clones 60 and 14-44 showed that the 26-bp increase in size for clone 60 was either the result of a duplication of 26 bp or a deletion of 26 bp in clone 14-44. Furthermore, the 26-bp duplication or deletion was bounded by a repeated hexanucleotide (TTCTCG) (see Fig. 5). Thus, the sequences can be represented as “abcda” and “abcda.” In addition, a C to T transition was found in the duplicated region, and clone 70 had the same duplication and transition as clone 60.

Clone 70 had a deletion of 46 bp with respect to clones 14-44 and 60, or clones 14-44 and 60 had a duplication of 46 bp with respect to clone 70. Seven bp were altered in the duplicated region in both clones 60 and 14-44. A similar oligonucleotide (TGGCT, TGCGCT, and TGGCGT) bounded the duplication/deletion, perhaps analogous to the repeated hexanucleotide discussed above.

A similar duplication of structure “abcaba”
was previously found in clone 14-44 in the viral sequence adjacent to the 3' LTR (nt -90 to nt -58) (23). In this case a pentanucleotide (TCCCA) bounded a 13-bp oligonucleotide. By molecular cloning of circular SNV proviral DNA from SNV-infected chicken cells, we isolated an infectious SNV clone in which the fragment containing this viral sequence had a slightly different size (data not shown). Therefore, it is likely this repeated sequence could also be deleted or duplicated during the viral life cycle.

The presence of the repeated oligonucleotide bounding the duplicated/deleted sequences and the mechanism of viral DNA synthesis with its requirement for "jumping synthesis" to make the LTRs indicate that a similar "slippage or jumping synthesis" might give rise to the deletions/duplications (25). Alternatively, homologous recombination might be the source of the variation. In cloned murine sarcoma virus and Rous-associated virus-2 DNA, tandem duplications were also found (7, 14, 20).

A similar hypothesis has been proposed for small deletions in globin and interferon genes (8, 11). However, a computer search of DNA sequences found in cellular genes revealed a very low frequency of duplications of the form "abcdabcda" (data not shown).

The restriction enzyme cleavage site analysis (summarized in Fig. 7) and the DNA sequencing indicate a high frequency of bp variation (1 of 100 from restriction enzyme cleavage sites and 1 of 90 from comparison of duplicated regions). Presumably, this high frequency is related to the use of a single-stranded template and to a low fidelity of reverse transcription (1, 16), although infidelity of RNA synthesis is also possible.

The coincidence of bp changes in the sequences in the duplicated regions in different viruses (nt 78 to 123 in clones 14-44 and 60; nt

**FIG. 6.** Size difference of U3 regions of the LTR of unintegrated viral DNA from recombinant proviruses. Recombinants between clones 60 and 14-44 were prepared as described in Materials and Methods. Unintegrated viral DNA was isolated from chicken cells infected with DNA of clones 60 and 14-44 and the two recombinants and was digested with *AvaI* and *SacI*. The digests were analyzed in a 7% agarose-6% acrylamide gel. DNAs were transferred to nitrocellulose paper and hybridized with a *32*P-labeled LTR probe. The drawing below the picture shows the 3' and 5' LTR of unintegrated viral DNA and cleavage sites of *SacI* and *AvaI*. The numbers indicate the size in bp. The 3' *AvaI* fragment is given the size from proviral DNA. Unintegrated DNA is probably 2 bp longer (23).
between 150 and 151 in clones 60 and 70) indicates that the sequence of clone 60 was ancestral to clones 70 and 14-44 and then there was recombination or mutation.

This high frequency of variants does not directly reduce viability. Six of seven of these clones are infectious, and the region of clone 70 causing loss of infectivity is other than those studied here (17, 18). Thus, SNV can tolerate the high frequency of variants found in the regions analyzed here.

**ACKNOWLEDGMENTS**

We thank G. Hoffman and S. Hellenbrand for technical assistance, R. Fitts, J. O’Rear, I. Chen, C. Gross, and J. Mertz for useful comments on the manuscript, and W. Goad and M. Kanehisa of the Los Alamos Scientific Laboratories Sequence Analysis Library for help with the computer analysis of cellular genes.

This work was supported by National Cancer Institute Program Project CA-07175 and 22543. H. M. T. is an American Cancer Society Research Professor.

This paper is dedicated to Norman Davidson in honor of his 65th birthday.

**LITERATURE CITED**

8. Efstratiadis, A., J. W. Posakony, T. Maniatis, R. M. Lawn,
LONG TERMINAL REPEAT OF AVIAN RETROVIRUS


