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Genetics

Presence of retrovirus reverse transcriptase-related gene sequences in avian cells lacking endogenous avian leukosis viruses

(endogenous provirus/endogenous virus-negative chicken/*pol* genes/relaxed hybridization conditions/nucleotide sequence)

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ABSTRACT Using a molecularly cloned viral DNA probe representing the entire avian sarcoma virus (ASV) reverse transcriptase (*pol*) gene, we have detected related sequences in DNA preparations from two avian species, ev^- chickens and Japanese quail, previously demonstrated to lack all endogenous avian leukosis viruses. Nucleotide sequence homology was detected only when hybridization conditions, which allowed the formation of stable duplexes with as much as 30% base mismatch, were used. No sequence homology could be detected when stringent hybridization conditions were used. Nucleotide sequence analysis of a clone representing the major *pol*-specific *Eco*RI restriction fragment from ev^- chicken embryo fibroblasts revealed DNA homology as high as 72% and implied amino acid homology as high as 82% when compared to the sequence of the ASV strain Prague C *pol* gene. These data reveal the presence of retroviral *pol* gene sequences in avian cell lines that lack endogenous retrovirus sequences, suggesting that a reverse transcriptase-related gene exists in these cells as either part of a more distantly evolved retrovirus or a cellular gene.

Retroviruses are currently the only known genetic entity capable of coding for reverse transcriptase, an enzyme that catalyzes the conversion of a single-stranded RNA molecule into a colinear double-stranded DNA molecule. It has been proposed that RNA-dependent DNA polymerase activity may be a required cellular activity (1) and that retroviruses may have evolved to an independent genetic status from this normal cellular system of DNA-to-RNA-to-DNA information transfer (2). Several recently characterized genomic structures, including pseudogenes (3–8) and truncated small nuclear RNAs (9–11), have been associated with reverse transcription-like reactions in eukaryotic cells, suggesting a possible function for reverse transcriptase activity in normal cells. Furthermore, RNA-dependent DNA polymerase activity has been detected in a wide variety of uninfected vertebrate species, including the domestic chicken (12–19). The chicken contains at least 16 different endogenous proviruses (20, 21), many of which could code for such a cellular reverse transcriptase activity. In fact, the ubiquity of endogenous viruses among vertebrates argues that any such cellular activity is likely coded for by an endogenous provirus. However, a healthy, fertile chicken lacking all endogenous avian leukosis viruses (ev^-) has been bred (22), suggesting that if indeed a reverse transcriptase activity is required for normal development in the chicken, it is not coded for by any as yet identified endogenous viral gene or cellular gene.

In an attempt to determine whether any nucleotide sequences, even distantly related to the avian retrovirus *pol* gene, can be detected in avian cells, particularly those lacking endogenous retroviruses, we have utilized specific cloned viral DNA probes and relaxed hybridization conditions to screen for such sequences. In this report, we document the presence of *pol*-related sequences in both ev^- chickens and Japanese quail.

MATERIALS AND METHODS

Cloned Viral DNAs. Plasmid pCERT-98 is an expression vector containing the entire reverse transcriptase gene from the PrA strain of avian sarcoma virus (ASV) and was provided by B. DeLorbe (Molecular Genetics Inc.). Plasmid pUC-RT was generated by subcloning a *Hin*dIII/*Sal* I fragment from pCERT (representing 85% of the *RT* gene) into the vector pUC-9.

Preparation of DNA Probes. All Southern blot data, except the mapping of pUC-ev^--RT, were generated by using gel-purified DNA fragments as probes. pCERT-98 was digested with various restriction endonucleases, and the *pol*-specific fragments were isolated from all other plasmid sequences by electroelution onto DE-81 paper in agarose gels. The eluted DNA was concentrated by DE-52 chromatography and ethanol precipitation. The probes then were resuspended and labeled by nick-translation (23) to specific activities of 2×10^8 cpm/μg to 8×10^8 cpm/μg. CsCl gradient-purified plasmid pUC-RT was nick-translated directly and used for the mapping experiments on pUC-ev^--RT.

Preparation of Cellular DNAs. High molecular weight genomic DNA was extracted from 11-day-old White Leghorn chicken embryos (ev^+ CEF and ev^- CEF), from 8-day-old Japanese quail embryos, and from the chemically transformed quail fibroblast cell line QT_6. Homogenized tissues or cells were resuspended in 0.2 M Tris·HCl, pH 8.1/0.1 M EDTA/0.5% sodium dodecyl sulfate/500 μg of Pronase per ml and digested at 37°C for 12–16 hr. The DNA was extracted several times with phenol/chloroform, 1:1 (vol/vol) and then chloroform. High molecular weight DNA was spooled out of solution after addition of sodium acetate to a final concentration of 0.2 M and 2 vol of ethanol. All DNAs were RNase treated, reextracted with phenol/chloroform and reprecipitated with ethanol prior to analysis.

Nucleic Acid Hybridization. DNAs were digested with restriction endonucleases, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose filter paper by a modification of the Southern procedure (24). The nitrocellulose filters to which DNA was bound were prehybridized for 16–20 hr at 43°C in 28% (vol/vol) formamide for less-stringent blots or 50% (vol/vol) formamide for stringent blots in 1 M NaCl/20 mM sodium phosphate, pH 6.8/0.1% sodium dodecyl sulfate/0.1% polyvinylpyrrolidone/0.1% Ficoll/0.1% bovine serum albumin/500 μg of boiled salmon sperm DNA per ml. Hybridizations were done at 43°C for 16–20 hr in the same solution used for prehybridization with the

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Abbreviations: ASV, avian sarcoma virus; CEF, chicken embryo fibroblasts; kb, kilobase(s); bp, base pairs.

addition of dextran sulfate to a final concentration of 10% (wt/vol) and nick-translated ^{32}P-labeled probe (3×10^6 cpm/ml). All blots were initially washed three times in 0.3 M NaCl/30 mM sodium citrate, pH 7.4/0.1% sodium pyrophosphate/0.1% sodium dodecyl sulfate at room temperature. The stringent blots were washed a fourth time in 60 mM NaCl/6 mM sodium citrate/0.1% sodium pyrophosphate/0.1% sodium dodecyl sulfate at 50°C for 3 hr and a fifth time in 75 mM NaCl/7.5 mM sodium citrate/0.1% sodium pyrophosphate/0.1% sodium dodecyl sulfate for 30 min at 67°C. The less-stringent blots were washed a fourth time only in 0.45 M NaCl/45 mM sodium citrate/0.1% sodium pyrophosphate/0.1% sodium dodecyl sulfate at 50°C for 3 hr. The filters then were dried and exposed to x-ray film.

Cloning of DNA in Phage λ gt10. High molecular weight DNA extracted from ev⁻ chicken embryos was digested to completion with *Eco*RI and fractionated on a 0.7% agarose gel. DNA fragments, ranging in size from 0.5 to 2 kb, were eluted onto DE-81 paper and purified. An aliquot of this ev⁻ CEF DNA (0.1 μg) was ligated to 1.9 μg of *Eco*RI-digested λ gt10 vector DNA (25) in 10 μl of ligation buffer (70 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/5 mM dithiothreitol/1 mM ATP) for 6 hr at 14°C. The ligation mixture was directly packaged into phage coats as described by Maniatis *et al.* (26). The resulting phage was plated on C600 Hfl cells, and nitrocellulose filter lifts were made from the plates and hybridized as for Southern filters (see above). Plaques showing hybridization were picked and plaque-purified three times. Plate lysates were used to prepare DNA for analysis (26).

DNA Sequencing. Cesium chloride gradient-purified plasmid (pUC-ev⁻-RT) DNA was digested with restriction enzymes, which generate a 3′ recessed end. The fragments were labeled with [^{32}P]dNTPs and DNA polymerase I Klenow fragment, purified on G-50 Sephadex columns, and then subcut with additional restriction endonucleases. Radiolabeled DNA fragments suitable for sequencing were obtained by electrophoresis on 5% polyacrylamide gels and purified by the crush-elution method (26). The fragments were sequenced by the chemical modification technique of Maxam and Gilbert (27). Sequence determinations were run on polyacrylamide gels of 8% and 20%, which then were exposed to x-ray film at −20°C.

RESULTS

Reverse Transcriptase-Related Sequences in Avian DNAs. Using the Southern blot hybridization procedure (24) and an ASV-derived probe that contained only reverse transcriptase nucleotide sequences, we screened DNA from a pair of avian species for the presence of reverse transcriptase-related nucleotide sequences. To compensate for considerable sequence divergence (i.e., 26–30% base mismatch), we incubated probes with filter-bound DNAs at a low formamide concentration in order to favor the formation of less-stable duplexes between the probe and distantly related cellular sequences. Under these hybridization conditions, sequence relationships detected between evolutionarily divergent species closely reflects true homologies as determined by nucleotide sequence anlysis (28–32).

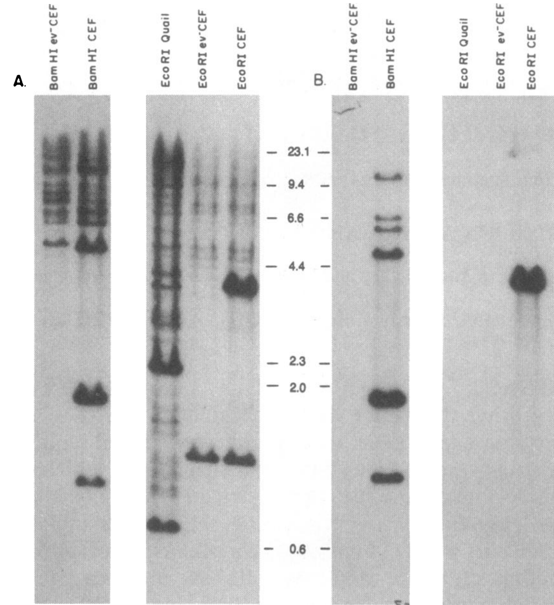


FIG. 2. Reverse transcriptase-related sequences in chicken and quail DNA. DNAs extracted from normal chicken embryos, ev⁻ chick embryos, and quail embryos were digested with *Eco*RI and *Bam*HI. Ten micrograms of DNA was loaded per channel and fractionated by agarose gel electrophoresis. The DNAs were transferred to nitrocellulose filters and hybridized to a nick-translated *pol*-specific probe under less-stringent (30% formamide) (*A*) and stringent (50% formamide) (*B*) hybridization conditions. Size markers are in kbp and represent *Hin*dIII-digested phage λ DNA.

A 2.7-kilobase (kb) restriction fragment specific for the ASV reverse transcriptase gene (Fig. 1) was isolated from the plasmid pCERT-98 and labeled by nick-translation. The probe was hybridized to filters containing *Eco*RI- and *Bam*HI-digested DNAs extracted from normal chicken embryos, ev⁻ chicken embryos, and quail embryos under both stringent and less-stringent hybridization conditions. Under stringent hybridization conditions (Fig. 2*B*), the only bands detected are the *pol*-specific bands expected from the endogenous avian leukosis viruses in normal chicken embryos. Neither the ev⁻ CEF DNA nor the Japanese quail DNA revealed any detectable sequence homology to the probe under these conditions, a result consistent with previous findings, indicating the lack of endogenous avian leukosis viruses in these particular cell types (22).

When less-stringent hybridization conditions were used (Fig. 2*A*), both the normal CEFs and the ev⁻ CEFs revealed an identical pattern of positively hybridizing restriction fragments that were not detected previously. The fragments

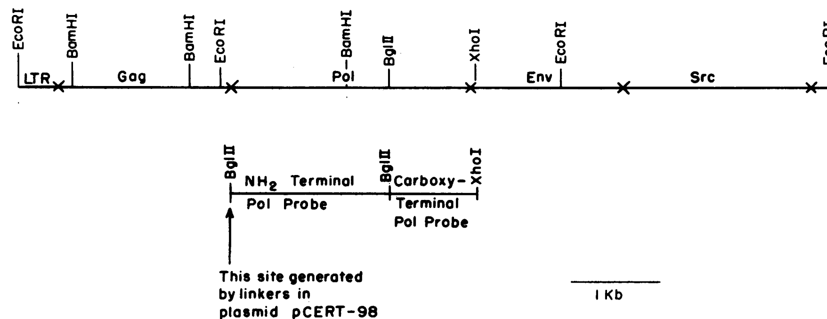


FIG. 1. Regions of the ASV genome represented by *pol*-specific probes. The restriction endonuclease map and the corresponding genetic map of the Prague A strain ASV genome are shown. The regions represented by the complete *pol* probe and the amino- and carboxyl-terminal-specific probes are outlined below.

detected consisted of one low molecular weight fragment (1.3 kb) and six high molecular weight fragments (5–15 kb). The *pol*-related fragment patterns were identical for all chicken samples when either *Eco*RI or *Bam*HI were used to digest the DNAs. The Japanese quail embryo DNA also revealed a new array of *pol*-related restriction fragments that were not detected under more stringent hybridization conditions. The chemically transformed quail fibroblast cell line QT_6 also was screened with the *pol* probe under relaxed hybridization conditions (data not shown). Both of the quail DNA samples gave hybridization patterns identical with one another when digested with either *Eco*RI or *Bam*HI. Thus, as observed with the CEF DNA, the *pol*-related sequences appear to be stably maintained at the same genetic locus in these two different genetic environments.

Reverse Transcriptase-Related Sequences in CEF DNA Hybridize Differentially to Amino-Terminal and Carboxyl-Terminal Regions of the *pol* Probe. Most of the eukaryotic protein-coding genes described to date have been shown to be split, consisting of protein coding sequences or exons separated from one another by noncoding sequences or introns (33). The cellular homolog to the viral *src* gene, termed proto-*src*, contains introns similar to most other eukaryotic protein-coding genes (34). On the other hand, retrovirus genes, with the exception of the *env* and *src* genes (35) in which a splicing reaction positions 397 nucleotides at the 5′ end of their mRNAs, do not contain introns. Thus, one possible way to distinguish between putative cell progenitors of retrovirus genes and more distantly evolved endogenous retroviruses would involve determining whether or not the avian leukosis virus-related sequences detected under low-stringency hybridization are split. In an effort to distinguish between these possibilities, amino-terminal- and carboxyl-terminal-specific regions of the reverse transcriptase gene were utilized as probes to see if the different reverse transcriptase-related restriction fragments observed in cell DNA would hybridize differentially to different regions of the reverse transcriptase gene. The original 2.7-kb probe was digested with the restriction endonuclease *Bgl* II to generate a 1.7-kb amino-terminal restriction fragment and a 1.0-kb carboxyl-terminal restriction fragment (Fig. 1). *Eco*RI-digested DNA from uninfected CEFs was analyzed with each probe separately under relaxed hybridization conditions (Fig. 3). The 1.7-kb amino-terminal probe hybridized extensively to the six high molecular weight reverse transcriptase-related fragments described above as well as to the endogenous viral sequences; however, hybridization to the low molecular weight fragment was barely detectable. Conversely, the 1.0-kb carboxyl-terminal probe hybridized extensively to the low molecular weight reverse transcriptase-related fragment but failed to hybridize to any detectable level to the high molecular weight fragments. This probe also detected the endogenous virus reverse transcriptase fragments as expected. The same experiment was done with DNA from the ev⁻ chick embryos, and an identical result was obtained with the exception that no endogenous viral sequences could be detected (Fig. 3). These data suggest that the various reverse transcriptase-related fragments detected by low-stringency hybridization represent different regions of the reverse transcriptase gene spread over a large region of DNA.

Molecular Cloning and Mapping of the 1.3-kb *pol*-Related Fragment from ev⁻ CEFs. Utilizing the λ phage insertion vector λ gt10, we attempted to clone the low molecular weight (1.3 kb) *pol*-related fragment from the ev⁻ CEFs. High molecular weight DNA was extracted from ev⁻ chicken embryos and digested to completion with *Eco*RI; 50 μg of this DNA was then loaded onto a preparative agarose gel. A region of the gel encompassing those DNA fragments, rang-

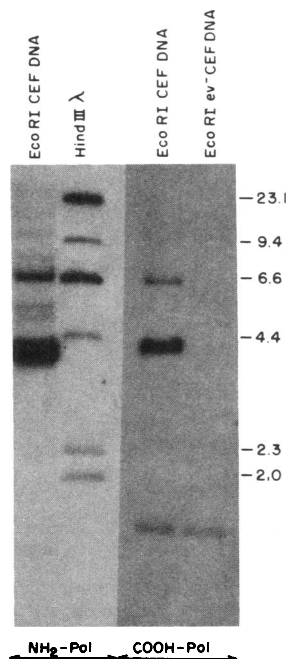
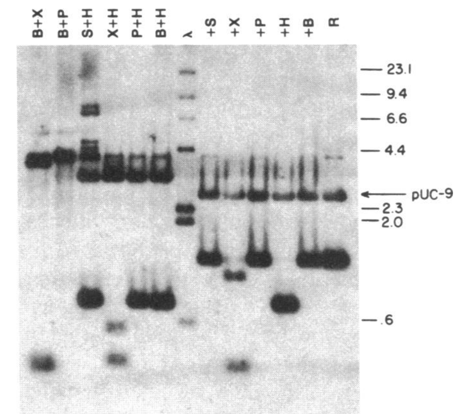


FIG. 3. Amino-terminal and carboxyl-terminal reverse transcriptase-related sequences in chicken DNA. Ten micrograms of each DNA was digested with *Eco*RI and then hybridized under less-stringent conditions to probes representing the amino terminus of the *pol* gene (far left lane) and the carboxyl terminus of the *pol* gene (right two lanes). The lane labeled *Hin*dIII λ contains ³²P-labeled *Hin*dIII-digested phage λ DNA.



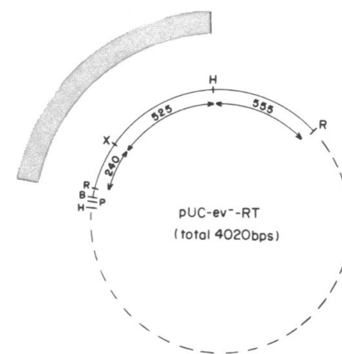


FIG. 4. Restriction enzyme and Southern blot analysis of plasmid pUC-ev⁻-RT. Equal amounts of pUC-ev⁻-RT (0.2 μg) were treated with a variety of enzymes (identified above each lane) in single (R lane only) and double digests (all other lanes), fractionated by electrophoresis, and transferred to nitrocellulose. The filter was hybridized with the nick-translated viral-specific *pol* fragment cloned into pUC-9 (pUC-RT) under relaxed hybridization conditions. The enzymes used were: R, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst* I; X, *Xho* I; S, *Sac* I. (Those lanes marked with an initial "+" and a single enzyme were digested in a combination of *Eco*RI and the enzyme designated.) The markers represent ³²P-labeled *Hin*dIII-digested phage λ. The portion of the 1.3-kb cloned insert that hybridizes to the probe is indicated by shading.

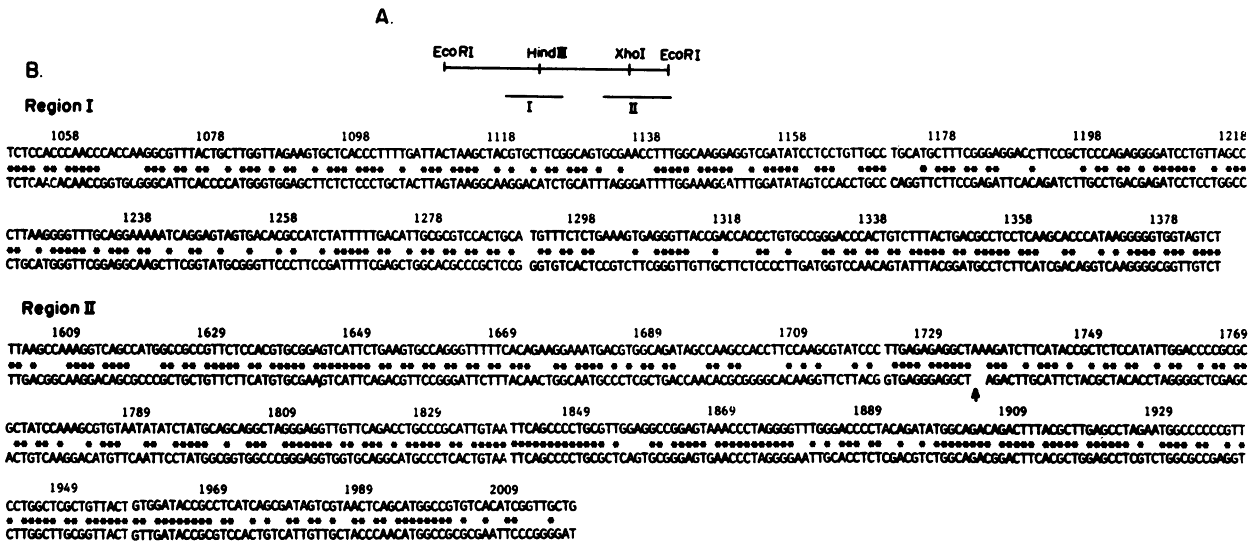


FIG. 5. Nucleotide sequence comparison of the ev$^-$ CEF *pol*-related fragment with the PrC ASV reverse transcriptase gene. (*A*) Two distinct regions of the pUC-ev$^-$-RT plasmid insert that were sequenced. (*B*) Sequences are aligned to give the best fit. The upper line of each row represents the PrC ASV gene sequence and the lower line represents the sequence derived from pUC-ev$^-$-RT. The numbering corresponds to the PrC ASV sequence with position 1 being the first base of the *pol* gene. A 2-bp gap is introduced into the pUC-ev$^-$-RT at position 1734 to allow for maximum alignment. Identical bases are indicated by an asterisk.

ing in size from 0.5 to 2 kb, was eluted from the gel and ligated with *Eco*RI-digested λ gt10. The recombinant molecules then were packaged *in vitro*. The resulting phage population was screened with the entire *pol*-specific probe under relaxed hybridization conditions. Positive plaques were subjected to several rounds of plaque purification before DNA was prepared from one of the clones. *Eco*RI digestion of the DNA from this clone, followed by Southern blot analysis, revealed the presence of the desired 1.3-kb *pol*-specific fragment. This fragment was subcloned into the bacterial vector pUC-9 for further characterization. The plasmid, denoted pUC-ev$^-$-RT, was mapped with a variety of restriction endonucleases, and a rough determination of the *pol*-related region was determined by Southern blot analysis (Fig. 4) with the pUC-RT plasmid as a probe. This data revealed that the *pol*-related portion of the clone, as determined by this analysis, was restricted to a 765-bp *Eco*RI/*Hin*dIII fragment. The remaining portion of the clone revealed no detectable hybridization to the probe.

Nucleotide Sequence Analysis of the 1.3-kb ev$^-$ CEF *pol*-Related Fragment. As a means of determining more precisely the *pol*-related portion of the cloned 1.3-kb fragment, we sequenced two different regions of the clone, utilizing the base-specific chemical cleavage method of Maxam and Gilbert (27). The regions sequenced are shown in Fig. 5*A* and span 335 bp and 408 bp in regions I and II, respectively. Region II (Fig. 5*B*) shares 67% nucleic acid homology with the 3′ end of the PrC ASV reverse transcriptase gene, spanning the junction between the purported polymerase- and endonuclease-encoding regions of the gene. When the predicted amino acid sequences are compared, the two sequences reveal 72% homology over the entire 138 amino acids (Fig. 6). If conservative amino acid substitutions are allowed, the homology increases to 82% overall. However,

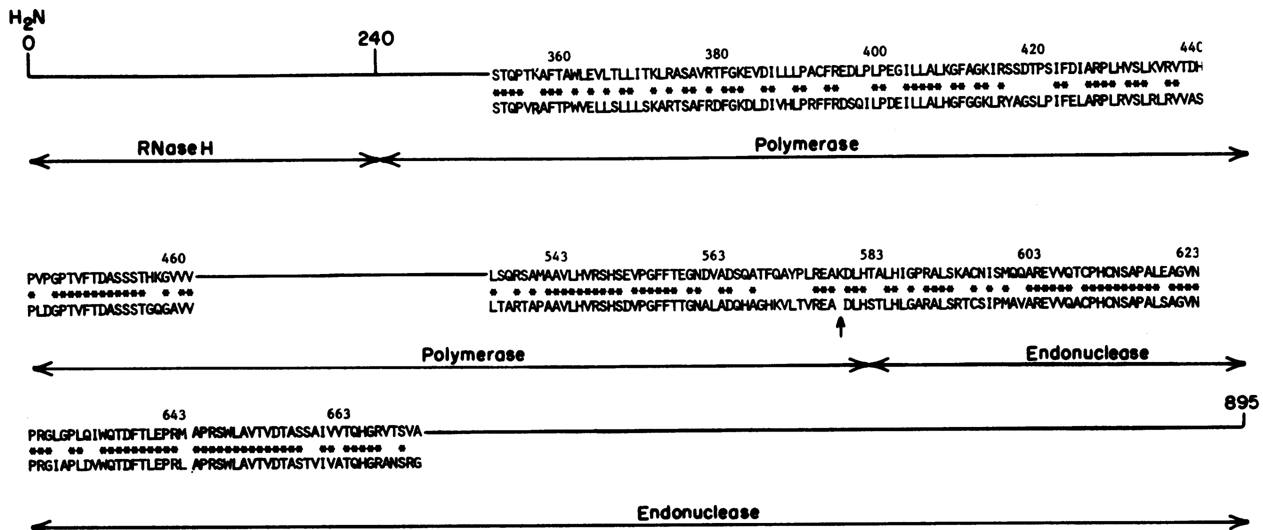


FIG. 6. Comparison of the deduced amino acid sequences encoded by the PrC ASV *pol* gene and the ev$^-$ *pol*-related fragment. The predicted amino acid sequences are compaired by using the standard single letter code. The upper line of each row represents the viral *pol* sequence and the lower line represents the ev$^-$ *pol*-related sequence. The regions of the gene coding for the RNase H, polymerase, and endonuclease activities are shown below each row. The numbering corresponds to the amino acids of the viral reverse transcriptase. Identical amino acids are indicated by an asterisk, and the arrow denotes the position of the gap introduced for maximum alignment.

because of a 2-base-pair (bp) deletion at position 132 of the pUC-ev$^-$-RT nucleotide sequence, the predicted amino acid translation would fall out of frame at amino acid 44 and would soon terminate, generating a truncated protein, interestingly, the frame shift and termination would occur just after the polymerase–endonuclease junction, suggesting that only the endonuclease domain would be lost. Region I (Fig. 5*B*) shares 57% nucleotide sequence homology with the viral *pol* gene at a position located 200 bp upstream from the sequence homologous to region II. This corresponds to the purported polymerase-encoding domain of the reverse transcriptase gene. The predicted amino acid sequence from this stretch suggests an open reading frame over the entire 111 amino acids with an overall homology of 59% with that of PrC ASV reverse transcriptase (Fig. 6). If conservative amino acid substitutions are allowed in this region, the homology increases to 77%.

DISCUSSION

In this study, we have shown that nucleotide sequences related to the avian retroviral reverse transcriptase gene can be detected in the genomic DNAs from a variety of avian species, including those that apparently lack related endogenous retroviruses. That the observed nucleotide sequence homology was indeed reverse transcriptase-specific rather than fortuitous hybridization was demonstrated by molecularly cloning one of the *pol*-related restriction endonuclease DNA fragments and subjecting it to nucleotide sequence analysis. The results of this analysis revealed significant nucleic acid homology with the Rous sarcoma virus *pol* gene, confirming the purported relatedness suggested by Southern blot hybridization under conditions of relaxed stringency.

The possible origin and function of these sequences remains to be determined; however, the sequences detected probably represent either distantly related endogenous proviruses that were introduced into the ancestral germ lines of all of the species by exogenous viral infection or cellular genes that share limited sequence homology to the avian retroviral reverse transcriptase gene. Although our data do not distinguish between these possibilities at this time, several interesting points can be made concerning this issue. For instance, identical blot hybridization patterns were detected in the genomic DNAs from QT_6 cells and Japanese quail embryos, suggesting an evolutionarily conserved genetic locus. The same result was observed in the various chicken DNA samples examined, including the DNA from two ev$^-$ chickens, which have undergone extensive crossbreeding to rid them of any of the previously defined endogenous proviral sequences. The hybridization pattern obtained when amino-terminal-specific and carboxyl-terminal-specific probes were used could reflect the presence of introns within the *pol* gene-related sequences analyzed, suggesting a cellular origin for the reverse transcriptase gene. On the other hand, the hybridization pattern could represent multiple proviruses located at different positions within the genome. This possibility would require that every provirus have a deletion in the amino-terminal or carboxyl-terminal region of the *pol* gene and that these deletions all occur specifically at the site we chose to split the gene into amino- and carboxyl-terminal probes.

Probably the most convincing method of distinguishing the origin of these *pol*-related sequences would be to determine whether additional retrovirus gene sequences, such as *gag*- and *env*-related sequences, can be detected within the genomes of these avian species. If the sequences identified in this study represent more distantly related endogenous proviruses, we would expect to find *gag*- and *env*-related sequences linked to the *pol*-related sequences in the order *gag-pol-env*. If, however, these sequences represent cellular genes, we would expect to find them located at different genetic loci. The identification and molecular cloning of *gag*- and *env*-related sequences from these avian cells should allow us to determine unequivocally if these sequences more closely resemble endogenous proviruses or cellular genes.

Note Added in Proof. Preliminary low-stringent hybridization with *env*- and *gag*-specific probes to *ev*$^-$ chicken DNA indicates the presence of these sequences in this avian species. Moreover, analysis of the *gag*-related sequences suggests linkage to the *pol*-related sequences reminiscent of an endogenous provirus.

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