Sequences near the 5' Long Terminal Repeat of Avian Leukosis Viruses Determine the Ability To Induce Osteopetrosis

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Avian leukemia virus (ALV)-induced osteopetrosis is associated with the accumulation of unintegrated viral DNA in osteoblasts. Viruses constructed from the DNAs of an osteopetrotic-inducing ALV (Br21) and a non-osteopetrotic-inducing ALV (RAV-0) have been used to test for the role of viral genes in the induction of osteopetrosis. Our results map osteopetrotic potential to a 1,400-base-pair region near the 5' long terminal repeat. This region contains signals for the splicing, translation, and packaging of viral mRNAs and coding sequences for the gag proteins p19 and p10 and the N terminus of p27.

Avian leukemia virus (ALV)-induced osteopetrosis results from viral infections causing the abnormal growth and differentiation of osteoblasts (for review, see reference 20). Viruses that cause osteopetrosis undergo an atypical life cycle involving the persistent synthesis of viral DNA in osteoblasts (15). This atypical life cycle results in osteopetrotic bone containing an average of 5 to 10 copies of unintegrated viral DNA per cell plus an additional 5 to 10 copies of integrated proviral DNA per cell (15). The persistent synthesis of viral DNA by osteopetrotic-inducing viruses is a tissue-specific phenomenon which occurs in osteoblasts but not in fibroblasts, bone marrow cells, or lymphoid cells (15). Also, the sex of a chicken influences susceptibility to ALV-induced osteopetrosis, with males being at a higher risk for the development of this disease than females (15).

All ALVs of exogenous origin appear to spawn variants that induce osteopetrosis. This fact is evidenced by the occurrence of viruses that induce intermediate- to rapid-onset osteopetrosis in virtually all laboratory stocks of ALVs (15, 21). We have previously shown that the ability to induce osteopetrosis is determined by sequences in the gag-pol-5' env region of the viral genome (18). In this study we have narrowed the region determining osteopetrotic potential to a 1,400-base-pair region near the 5' long terminal repeat.

MATERIALS AND METHODS

Recombinant DNAs. Lambda Br21 and pBR21 are lambda and pBR322 clones, respectively, of the DNA of a subgroup E ALV (Br21) that induces a high incidence of rapid-onset osteopetrosis (18). pRAV-0 is a pBR322 clone of the DNA of RAV-0, a non-osteopetrotic-inducing subgroup E ALV (18). The viral DNAs in lambda Br21, pBR21, and pRAV-0 are cloned at the Sall site at base pair ~6050 of Br21 and RAV-0.

Construction of recombinant viruses. Constructed viruses are designated RecALVs, with the prefix p (plasmid) or lambda indicating DNA in a cloning vector. Restriction endonuclease digestions, gel electrophoresis, recovery of fragments, and ligations were conducted as previously described (18). The pRecALV60 DNAs were constructed by ligating the 4.9-kilobase (kb) XbaI-Sall fragment of lambda Br21 with a 2.7-kb XbaI-Sall fragment of pRAV-0 linked to pBR322 (Fig. 1 and 2). Recombinants were analyzed for PsI fragments to verify the presence of the appropriate fragments of lambda Br21 and pRAV-0. The lambda recALVBr70 DNAs were constructed with a complete SacI-plus-XbaI digest of lambda Br21 to obtain the two phase arms linked to viral sequences. The internal 3.1-kb SacI-to-XbaI fragment of pRAV-0 was obtained by gel purification of a complete XbaI and partial SacI digest of pRAV-0. These three fragments were ligated and packaged in vitro, and recombinant plasmids were screened for diagnostic PsI fragments.

The pRecALVBr130 DNAs were prepared from the 10.6-kb SacI fragment of pBR21 containing the bulk of the viral DNA linked to pBR322 and the 1.4-kb internal SacI fragment of pRAV-0. After ligation of the two fragments and transformation of HB101, clones were screened for diagnostic PsI and Sall fragments. The pRecALVBr140 DNAs were prepared from a complete XbaI and partial SacI double digest of pBR21 to obtain the 10.3-kb fragment containing the bulk of viral DNA linked to pBR322. The internal 1.7-kb SacI-XbaI fragment of pRAV-0 was gel purified from a complete XbaI-SacI double digest of pRAV-0. After ligation of the two fragments and transformation of HB101, clones were screened for diagnostic PsI and Sall fragments.

Recovery of virus from molecularly cloned DNAs. DNAs encoding viral sequences were cleaved from the cloning vectors with Sall, self-ligated, and transfected into turkey cells for the recovery of infectious virus (18). Culture media from transfected turkey cells or from 15* ev1 chicken cells infected with virus produced by transfected turkey cells were used as virus stocks. Titers of virus stocks were determined by testing for the amount of particulate-RNA-directed-DNA polymerase (13) and multiplying this amount by the number of infectious units observed for 1 cpm of reverse transcriptase activity.

Pathogenicity tests. Viruses were tested for their pathogenic potential by the intravenous inoculation of ~106 infec-
tious units into day-old K28 chickens (18). Rapid-onset osteopetrosis appeared within 1 month after infection and reached its maximum incidence by 2 months after infection. Pathogenicity tests were terminated at 4 months after infection. Osteopetrosis was diagnosed by observing the leg bones of infected chickens.

RESULTS

gag-pol-5' env sequences and osteopetrotic potential. Sequences that determine osteopetrotic potential had been previously mapped to a 5.8-kb SacI-SalI fragment containing noncoding sequences that lie between the 5' long terminal repeat and gag, i.e., all of the viral gag and pol genes as well as the 5' end of the viral env gene (18). To test whether osteopetrotic potential was determined by sequences in the left or right half of this 5.8-kb region, the 3.1-kb SacI-XbaI fragment and the 2.7-kb XbaI-SalI fragment from sequences encoding a benign virus (pRAV-0) were substituted for the comparable fragments in pBr21 (Fig. 1 and 2). The DNA constructs containing gag and 5' pol sequences (SacI-XbaI fragment) of pRAV-0 were designated lambda RecALV70s, with independent isolates being designated lambda Br71 and lambda Br72. The constructs containing the 3' pol and 5' env sequences (XbaI-SalI fragment) of pRAV-0 were designated pRecALV60s, with independent isolates being termed pBr61 and pBr62. The structures of pBr61, pBr62, lambda Br71, and lambda Br72 were verified by mapping for diagnostic PstI sites (Fig. 1).

Viruses expressed by the pRecALV60s (Br61 and Br62) and the lambda RecALV70s (Br71 and Br72) were tested for disease potential by intravenous inoculation into day-old K28 chickens (Fig. 2). By 3 weeks, chickens infected with Br61 and Br62 exhibited distorted and enlarged leg bones. By 4 months after infection, 83% of the inoculated males and 22% of the inoculated females had developed moderate to severe cases of osteopetrosis. In the same period, only one of the males inoculated with Br71 or Br72 developed moderate to severe osteopetrosis. Some of the Br71- and Br72-inoculated chickens developed unusually thin leg bones. None of the Br61- and Br62-inoculated chickens developed unusually thin bones.

At 1 month after infection, sera from test birds were assayed for viremia by testing for the amount of particulate reverse transcriptase. All of the test groups exhibited comparable levels of viremia (Table 1). Since both the Br60s and Br70s viruses had grown well in chickens but only the Br60s had induced a high incidence of rapid-onset osteopetrosis, sequences that determine osteopetrotic potential must lie between bases 255 and 3378 of the genome of Br21 (Fig. 1). This region of the ALV genome contains 124 bases of 5' untranslated sequences and all of the gag gene as well as the 5' end of the pol gene.

FIG. 1. Restriction endonuclease maps of pRAV-0 and lambda Br21. The schematics display viral sequences as they would appear in the RNA form of the genome. Only sites that were used to construct and verify the genomes of the recombinant viruses are indicated. *, Restriction endonuclease sites that were used to distinguish fragments of pRAV-0 and lambda Br21 or pBr21. L, SalI; M, SmaI; P, PstI; S, SacI; X, XbaI.

gag and 5' pol sequences and osteopetrotic potential. The next series of constructs was designed to test whether osteopetrotic potential was determined by sequences in the left or right half of the 3.1-kb SacI-XbaI fragment. In these constructs, a 1.4-kb SacI-SacI fragment (5' gag sequences) and a 1.7-kb SacI-XbaI fragment (3' gag and 5' pol sequences) of pRAV-0 were substituted for the comparable fragments in pBr21 (Fig. 1 and 2). The constructs with 5' gag sequences of pRAV-0 were termed the pRecALV130s, with independent isolates being numbered pBr131 and pBr132. The constructs with the 3' gag and 5' pol sequences of pRAV-0 were named the pRecALV140s, with independent isolates being designated pBr141 and pBr142. The fragment content of pRecALV130s and pRecALV140s was verified by mapping for diagonal PstI and SmaI sites (Fig. 1).

Viruses recovered from pBr131, pBr132, pBr141, and pBr142 were tested for osteopetrotic potential by intravenous inoculation into day-old K28 chickens (Fig. 2). By 3 weeks postinoculation, the leg bones of the chickens inoculated with Br141 and Br142 exhibited signs of osteopetrosis. By 4 months after infection, 90% of the Br141- or 142-inoculated males and 42% of the Br141- or Br142-inoculated females had developed moderate to severe osteopetrosis. In the same time, none of the Br131- or Br132-inoculated chickens developed moderate to severe osteopetrosis. At 1 month postinoculation, test groups of Br131- or Br132-inoculated chickens had levels of viremia comparable to those in test groups of Br141- or Br142-inoculated chickens (Table 1). Thus, 5' sequences bounded by the SacI sites at

FIG. 2. Recombinant viruses: genomes and osteopetrotic potential. Schematics present the DNA form of the viral genomes. Only the restriction endonuclease sites used in the constructions are indicated. S, SacI; X, XbaI; L, SalI.
bases 255 and 1656 of the genome of Br21 confer the potential to induce a high incidence of osteopetrosis.

Verification of viruses in test groups. Sera from chickens infected with the Br130s or Br140s were used to infect chicken embryo fibroblasts. Infected cultures were passaged two times, and DNA was prepared. These DNAs were tested for PstI and SmaI fragments that distinguish the genomes of the Br130s and Br140s viruses (Fig. 1 and 3). As expected, cells infected with virus recovered from chickens infected with Br141 or Br142 exhibited the 1.3-kb PstI fragment that is diagnostic for the 1.4-kb SacI fragment of Br21 and the 0.43-kb SmaI fragment that is diagnostic for the 1.7-kb SacI-XbaI fragment of pRAV-0. DNAs isolated from the osteopetrotic bones of Br141- or Br142-infected chickens also exhibited these diagnostic fragments. DNAs isolated from cells infected with virus recovered from Br131- and Br132-infected chickens did not exhibit the 1.3-kb PstI and 0.43-kb SmaI fragments. Thus, the test groups had been infected with the appropriate test viruses.

High copy numbers of viral and proviral DNA in Br141- and Br142-induced osteopetrosis. To test whether persistent synthesis of viral DNA was associated with osteopetrosis induced by Br141 and Br142, undigested DNAs from osteopetrotic bones were fractionated by electrophoresis through agarose and then analyzed for viral sequences by the Southern technique. Autoradiographs of these blots readily revealed the presence of unintegrated DNA in Br141- and Br142-induced osteopetrosis (Fig. 3B).

Estimates of the total amount of viral and proviral DNAs in Br141- and Br142-induced osteopetrosis were obtained from Southern blot analyses of EcoRI-digested DNAs. Densitometer tracings of bands diagnostic for comparable sequences in ev 1 (8.5-kb fragment) and Br141 or Br142 (2.4-kb fragment) were used to estimate the total amount of Br141 or Br142 DNA in cells (15). These estimates indicated that the osteopetrotic bones of Br141- and Br142-infected chickens contained 10 to 20 copies of viral and proviral DNAs per cell. This copy number is similar to that observed in cases of rapid-onset osteopetrosis induced by Br21 (15).

DISCUSSION

Our results map osteopetrotic potential to a 1,400-base-pair region between bases 255 and 1656 of the ALV genome. Since the induction of osteopetrosis is associated with the accumulation of viral and proviral DNAs, we propose that one or more functions in this region confer osteopetrotic potential by determining whether or not an infection will accumulate viral DNA. The viral DNA in osteopetrotic bone contains an unexpected 6.8-kb EcoRI fragment (15). This fragment is characteristic of virus that is undergoing reverse transcription (15). Therefore, the accumulation of viral DNA in osteopetrotic bone appears to be due to reverse transcription of viral RNA. At least two phenomena affect the reverse transcription of viral RNA in an infected cell: the establishment of superinfection resistance and the assembly of virions during budding.

Superinfection resistance and the control of viral DNA synthesis. In ALV infections, the establishment of superinfection resistance is mediated by newly synthesized envelope glycoproteins interfering with the activity of receptors for virus (24). Once interference is established, the efficiency of superinfection is 10-3 to 10-4-fold less than the efficiency of infection of normal cells. The establishment of superinfection resistance has never been directly correlated with cessation of viral DNA synthesis. However, this correlation seems likely, because the prevention of superinfection by neutralizing antibody prevents transient accumulations of viral DNA in ALV- and reticuloendotheliosis-infected cells (22).

The establishment of interference depends solely on the expression of envelope glycoproteins and does not require the expression of viral core proteins or the assembly of virus particles (14). Thus, mutations that affect the establishment of interference should lie in sequences that determine the structure or synthesis of envelope glycoproteins. At least two such functions reside in the region to which osteopetrotic potential has been mapped (17). The first function is codons for the first six amino acids of the env precursor protein. The second function is the splice donor used in the processing of env mRNA (11, 17). Sequences in this region may also affect the establishment of superinfection resistance by determining the efficiency of splicing of env mRNA (9a).
Assembly of virions and the control of viral DNA synthesis. RNA that is synthesized in the infected cell does not appear to be available for reverse transcription until it has been assembled into a virus particle during budding. One explanation for this finding has been that the precursor to reverse transcriptase is not a polymerase until it has been cleaved to its mature form during budding (23, 26). However, recent experiments with a mutant of Moloney murine leukemia virus suggest that the pol precursor may have high reverse transcriptase activity (4). Alternatively, assembly of reverse transcriptase and viral RNA into a virion may confer a spatial arrangement that facilitates the synthesis of viral DNA. Circumstantial support for this possibility is found in the apparent reverse transcription of newly synthesized RNA in cells infected with a virus that undergoes intracellular assembly into A-type particles (12) and in the requirement for the maintenance of at least some virus structure for the reverse transcription in vitro of full-length viral DNA (1, 8, 16).

Sequences that determine osteopetrotic potential contain several functions that affect the assembly of virus particles. Among these functions are cis-acting signals which influence the efficiency with which viral RNA is packaged into virions (for reviews, see references 2 and 3) and sequences encoding the N terminus of the p27 protein which forms the shell surrounding the viral nucleoprotein (for reviews, see references 5 and 6).

Accumulation of viral DNA as a mechanism for retrovirus-induced disease. The association of unintegrated viral DNA with disease is not unique to ALV-induced osteopetrosis. Unintegrated viral DNA has been observed in the bone marrow of cats infected with feline leukemia viruses that cause feline acquired immune deficiency syndrome (AIDS) (10). Unintegrated viral DNA has also been found in cells that are undergoing cytopathic effects after infection with ALVs (25), reticuloendotheliosis virus (9), visna virus (7), and human AIDS-associated viruses (19). In the case of ALV-induced osteopetrosis, unintegrated viral DNA is associated with cell dysfunction, whereas in feline as well as human AIDS, such DNA may be associated with the death of the infected cell. Interestingly, in cytopathic ALV and reticuloendotheliosis virus infections, 5 to 10 times more unintegrated viral DNA accumulates than in osteopetrotic bone (15, 22). Thus, the amount of unintegrated ALV DNA may determine whether an infected cell malfunctions or dies.

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LITERATURE CITED


