Molecular Cloning of the Avian Erythroblastosis Virus Genome and Recovery of Oncogenic Virus by Transfection of Chicken Cells

BJÖRN VENNSTRÖM,^{1*} LOIS FANSHIER,¹ CARLO MOSCOVICI,² AND J. MICHAEL BISHOP¹

Department of Microbiology and Immunology, University of California, San Francisco, California 94143,¹ and Veterans Administration Hospital, Gainesville, Florida 32601²

Avian erythroblastosis virus (AEV) causes erythroblastosis and sarcomas in birds and transforms both erythroblasts and fibroblasts to neoplastic phenotypes in culture. The viral genetic locus required for oncogenesis by AEV is at present poorly defined; moreover, we know very little of the mechanism of tumorigenesis by the virus. To facilitate further analysis of these problems, we used molecular cloning to isolate the genome of AEV as recombinant DNA in a procaryotic vector. The identity of the isolated DNA was verified by mapping with restriction endonucleases and by tests for biological activity. The circular form of unintegrated AEV DNA was purified from synchronously infected quail cells and cloned into the *EcoRI* site of λ gtWES-B. A restriction endonuclease cleavage map was established. By hybridization with complementary DNA probes representing specific parts of avian retrovirus genomes, the restriction map of the cloned AEV DNAs was correlated with a genetic map. These data show that nucleotide sequences unique to AEV comprise at least 50% of the genome and are located approximately in the middle of the AEV genome. Our data confirm and extend previous descriptions of the AEV genome obtained by other procedures. We studied in detail two recombinant clones containing AEV DNA: the topography of the viral DNA in the two clones was virtually identical, except that one clone apparently contained two copies of the terminal redundancy that occurs in linear viral DNA isolated from infected cells; the other clone probably contained only one copy of the redundant sequence. To recover infectious virus from the cloned DNA, we developed a procedure for transfection that compensated for the defectiveness of AEV in replication. We accomplished this by ligating cloned AEV DNA to the cloned DNA of a retrovirus (Rous-associated virus type 1) whose genome could complement the deficiencies of AEV. Ligation of the two viral DNAs was facilitated by using a neutral fragment of DNA as linker between otherwise noncompatible termini. Cloned AEV DNA gave rise to infectious AEV capable of transforming fibroblasts and bone marrow cells in culture and of inducing both sarcomas and erythroleukemia in chickens. We conclude that the cloned DNAs represent the authentic genome of AEV undisturbed by the cloning procedure. Molecular cloning offers a powerful approach to the identification and characterization of retrovirus genomes.

A set of rapidly acting leukemia viruses has been isolated from chickens in the field (13). Each of these viruses requires a second, "helper" virus for replication, and the entire set of viruses has, therefore, been denoted as defective leukemia viruses. The defective leukemia viruses of chickens have been divided into three main groups in accordance with their target cell specificities for transformation and on the basis of nucleic acid homologies among their putative oncogenes (4, 13, 32).

(i) Viruses of the myeloblastosis group comprise two isolates, avian myeloblastosis virus and E26, which rapidly induce myeloblastosis in

chickens and transform cells of myeloid origin in vitro. The nature of the defectiveness in replication of these viruses is unknown, since their genomes have yet to be fully characterized.

(ii) The myelocytomatosis viruses are represented by four different field isolates (MC29, MH-2, CM II, and OK10), which may induce carcinomas, sarcomas, or myelocytomatosis in fowl, and which transform fibroblasts and macrophages in culture. The haploid genomes of these viruses are generally 5.5 to 6.0 kilobases (kb) in length (11). Mapping by analysis of oligonucleotides (11) and of heteroduplexes (24) has identified a unique nucleotide sequence that

is alleged to be responsible for oncogenesis by the virus; this sequence is located in the middle of the viral genome, replacing all of *pol* and part of both *gag* and *env*, the three replicative genes of retroviruses (5).

(iii) The avian erythroblastosis viruses (AEV) are presently represented by two isolates that are identical in all properties tested to date (13). Erythroblastosis develops in 1 to 2 weeks after intravenous injection of virus, although sarcomas may also occur when intramuscular administration is used to delay the onset of the rapidly fatal erythroblastosis. AEV transforms both fibroblasts and cells of erythroid origin in vitro (14, 15).

The genome of AEV is a 5.0- to 6.0-kb RNA with a contiguous sequence of around 3.0 kb that is unique to AEV and is located in the middle of the genome (6, 26, 27); the remainder of the genome is similar to that of MC29. Three lines of evidence suggest that the oncogene of AEV may harbor more than one functional domain.

(i) Two proteins have been identified that may be encoded in the AEV genome. The first has been found in infected cells, has a molecular weight of 75,000, and is apparently encoded by both the portion of *gag* retained in AEV and adjacent nucleotide sequences that are part of the alleged oncogene (19, 20). The second protein, with a molecular weight of 40,000, has been produced by translation of viral RNA in vitro and is thought to be encoded entirely by the oncogene (28, 45), but has yet to be detected in infected cells. To date, neither of these proteins has been directly implicated in neoplastic transformation.

(ii) Cells transformed by AEV contain two AEV-specific mRNA's (D. Sheiness, B. Vennström, L. Fanshier, C. Moscovici, and J. Michael Bishop, manuscript in preparation). One of these is the size of the viral genome and is apparently the mRNA for the 75,000-dalton protein (28, 31); the other is smaller than the viral genome, carries a portion of the oncogene not represented in the 75,000-dalton protein (Sheiness et al., in preparation), and probably gives rise to a 40,000-dalton protein (unpublished data of M. Prival-sky).

(iii) Graf and his colleagues have described both conditional and nonconditional mutants of AEV that have lost the capacity to transform erythroid cells, but remain capable of inducing part or all of the transformed phenotype in fibroblasts (12, 33). The sites of these mutations have not yet been mapped on the viral genome.

The genetic and biochemical analyses of AEV have been hampered by the genetic defectiveness of the virus and by difficulties encountered in preparing practicable quantities of the viral

genome. To circumvent these impediments, we used molecular cloning to isolate and propagate the genome of AEV in the form of biologically active DNA. We mapped the topography of the viral DNA by the use of restriction endonucleases, and we were able to correlate these results with the genetic map of the avian sarcoma virus (ASV) genome. Our work required that we develop a new procedure for transfection of the DNA of genetically defective retroviruses. The results presented here will serve as the departure point for identification and characterization of the AEV oncogene by site-specific mutagenesis. Moreover, the isolated viral DNA and the subclones derived from it provide a practical source of rigorously defined reagents for use in the characterization of viral mRNA's and in the exploration of the evolutionary origins of the AEV oncogene.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts and QT-6 cells (a quail cell line derived from a chemically induced fibrosarcoma) (30) were grown as previously described (34). AEV strain ES4 (subgroup B) was obtained from C. Moscovici. To produce an AEV stock of subgroup A that would infect quail cells, chicken embryo fibroblasts, C/E, were infected with AEV-ES4 and MCAV (the subgroup A helper of MC29), and the harvested virus was passaged in C/B cells. Transformation of fibroblasts and bone marrow cells was measured according to previously described procedures (15).

Preparation of supercoiled viral DNA. QT-6 cells were infected at a high multiplicity with AEV (MCAV). The high-molecular-weight cellular DNA was separated from low-molecular-weight DNA by Hirt fractionation 36 to 48 h after infection (17, 22, 35). DNA in the supernatant was extracted with phenol-chloroform and precipitated with ethanol. This DNA was further subjected to extraction with phenol equilibrated to pH 4.0. During this procedure, the remaining high-molecular-weight linear DNA precipitates, lower-molecular-weight linear DNA denatures, and supercoiled DNA stays intact in solution (46; DeLorbe and Vennström, unpublished data). Supercoiled DNA and denatured linear DNA were recovered by ethanol precipitation. Supercoiled DNA was further purified from low-molecular-weight nucleic acid by exclusion chromatography on Bio-Gel A50m agarose columns.

DNA cloning. Supercoiled AEV DNA from 3×10^8 QT-6 cells was cloned using λ gtWES-B as vector (29). Supercoiled AEV DNA, cleaved to linear form with *EcoRI*, was ligated to purified arms of vector DNA and packaged into phage in vitro according to the protocol of Blattner et al. (7). Phage was plated on *Escherichia coli* DP50 *supF*; plaques formed by recombinant phage were detected as described by Benton and Davies (3) with a ^{32}P -labeled complementary DNA (cDNA) probe enriched for sequences unique to AEV. Positive plaques were picked and purified. Phage DNA was prepared from 2-ml lysates, digested with *EcoRI*, and analyzed by electrophoresis in aga-

rose gels. Two recombinant phage with inserts of expected size (5.0 to 5.5 kb) were grown in large-scale cultures and used for further study. AEV DNA cloned in λ gtWES was excised with *EcoRI* and purified by sedimentation in 15 to 30% sucrose gradients containing 0.5 M NaCl, 10 mM EDTA, and 20 mM Tris (pH 7.4) for 18 h at 27,000 rpm in an SW27 rotor, or by preparative agarose gel electrophoresis. Fractions containing AEV DNA were pooled and precipitated with ethanol. Rous-associated virus type 1 (RAV-1) DNA, cloned in λ gtWES-B at the *SacI* site, was purified by similar techniques. RAV-1 recombinant phage was a gift from G. Payne.

Hybridization reagents. [32 P]cDNA_{rep} was prepared with avian myeloblastosis virus reverse transcriptase, viral 35S RNA as template, and oligomers of calf thymus DNA as primers, as previously described (34).

[32 P]cDNA_{3'} was synthesized as described by Shank et al. (34) and Tal et al. (42).

[32 P]cDNA_{gag} was prepared with a 1.3-kb *Bam*HI fragment from cloned Schmidt-Ruppin ASV DNA; this fragment is located between 0.4 and 1.7 kb from the 5' end of the 9.4-kb Schmidt-Ruppin ASV genome and represents the 5'-terminal part of the *gag* gene (10). The DNA was denatured and transcribed into radioactive cDNA with reverse transcriptase as described elsewhere (25).

[32 P]cDNA_{env} was prepared as [32 P]cDNA_{gag} above, using as template a 1.6-kb *Pvu*II fragment of Schmidt-Ruppin ASV that spans the junction between *env* and *src*. This fragment is positioned between 6.2 and 7.8 kb from the 5' end of the ASV genome (10). In the present studies, it served as a probe for *env* because AEV does not contain *src* (40).

[32 P]cDNA enriched for AEV-specific sequences was a gift from D. Sheiness and was prepared according to a protocol described elsewhere (38).

[32 P]cDNA entirely specific for AEV (cDNA_{AEV}) was prepared with the 2.5-kb *Pvu*II fragment that comprises the bulk of the rightward half of cloned AEV DNA (see Fig. 2) and is composed exclusively of nucleotide sequences that are specific to the AEV genome. The fragment was isolated by cleaving cloned AEV DNA with both *Pvu*II and *Xho*I and then isolating the resulting fragments by preparative electrophoresis in agarose gels. The isolated fragment was transcribed into radioactive cDNA as described for [32 P]cDNA_{gag}.

Restriction endonuclease mapping. Restriction endonucleases were purchased from Biolabs Inc. and were used according to the supplier's instructions. DNA fragments were analyzed by agarose gel electrophoresis as described previously (21). DNA bands were visualized by staining with ethidium bromide and photographed under UV light (36), or were localized by Southern blotting and hybridization (39). DNA fragments were ordered by appropriate double digestions and subsequent agarose gel electrophoresis. Fragments produced by cleaving simian virus 40 and λ DNA with *Hind*III provided size markers.

Annealing conditions. Nitrocellulose filters carrying DNA purified from infected cells were incubated for 3 days at 41°C with 10⁶ cpm of [32 P]cDNA probes per ml in a buffer containing 50% formamide as de-

scribed by Shank et al. (34). Filters carrying microgram amounts of cloned DNA were incubated with 10⁵ cpm of [32 P]cDNA per ml at 68°C for 3 to 5 h. Filters were washed and subjected to autoradiography (34).

Analysis of viral RNA. Virus was pelleted from culture medium that had been collected at 2-h intervals; viral RNA was isolated by digestion with proteinase K in 0.1 M NaCl-10 mM EDTA-20 mM Tris (pH 7.4)-0.5% sodium dodecyl sulfate for 20 min at 37°C, followed by extraction with phenol. RNA was precipitated with ethanol, dissolved in distilled water, and analyzed by gel electrophoresis in agarose gels containing methylmercuric hydroxide (2). RNA in gels was transferred to activated diazobenzylloxymethyl (DBM) paper and hybridized with [32 P]cDNA probes as described by Alwine et al. (1).

Transfection of chicken cells with cloned AEV DNA. Enzymatic ligation was employed to generate a hybrid molecule composed of the following species of DNA: the cloned genome of AEV, excised from the vector by hydrolysis with *EcoRI*; the cloned genome of RAV-1, excised from the vector with *SacI*; and a 3.8-kb fragment of DNA derived from the *EcoRI* B fragment of λ gtWES-B by cleavage with *SacI*. All of the DNAs were purified by rate-zonal centrifugation before use. The AEV DNA, RAV-1 DNA, and lambda fragment were used in the molar ratios 4:4:1, respectively. Ligation was carried out for 18 h at 4°C in 66 mM Tris-hydrochloride (pH 7.4)-6.6 mM MgCl₂-10 mM dithiothreitol at a DNA concentration of 50 μ g/ml with 1 U of T4 ligase per 10 μ g of DNA. At the end of the incubation, sheared salmon sperm DNA was added, and the DNA was extracted two times with chloroform before transfection.

Chicken cells were transfected with 200 ng of ligated DNA and 10 μ g of salmon sperm DNA per 60-mm dish by the CaCl₂ precipitation technique of Graham and van der Eb (16) as modified by Stowe and Wilkie (41). Cells were treated with 30% dimethyl sulfoxide in transfection buffer for 3 min at 4 h after addition of DNA. The cells were passaged at 18 h after transfection, and an equal number of secondary cultures of chicken fibroblasts were added. The cultures were passaged every 3 to 4 days, using one third of the cells. The medium from the cultures was tested for release of particles with reverse transcriptase activity after the third and the seventh passages. A virus stock was collected after the eighth passage and used to infect fresh secondary cultures of chicken embryo cells; 2 $\times$ 10⁶ cells were seeded in 25 ml of harvested medium, and the medium was changed on the next day. These cells developed a morphology typical for AEV transformation after the third passage (see below).

Biological and physical containment. All packaging, growth, and DNA isolation involving recombinant phage were carried out in a P2 containment facility utilizing the EK2-certified vector λ gtWES-B and its certified host *E. coli* DP50 *supF* in accordance with the National Institutes of Health Guidelines for Recombinant DNA Research.

RESULTS

Identification and molecular cloning of the AEV genome. AEV contains several defec-

tive genes and thus requires a helper virus for propagation (14, 15). Preparations of AEV contain two species of viral RNA that have been characterized by analysis of heteroduplexes (27) and of T1 oligonucleotides (6). The larger species (ca. 8.5 kb) is apparently the genome of the helper virus; the smaller species (ca. 5.4 kb) is thought to be the genome of AEV. Our objective was to isolate and amplify the genome of AEV by the application of molecular cloning. To this end, it was first necessary to characterize the unintegrated viral DNA in cells acutely infected with AEV.

Circular DNA was prepared from QT-6 cells harvested at 36 h after infection with AEV. The detection of the supercoiled AEV DNA was augmented by extracting DNA in the Hirt supernatants with phenol at pH 4.0; this procedure removed linear DNA, thereby improving the resolution in gel electrophoresis and reducing the background when radioactive cDNA's were hybridized to filters containing DNA that had been transferred from agarose gels (39). When [32 P]cDNA_{rep} was used as radioactive probe, only helper virus supercoiled DNA was detected, even after extensive autoradiographic exposure (Fig. 1, lane 1). Since the extent of homology between ASV and AEV was unknown, a radio-

labeled cDNA enriched for AEV sequences was used to detect the AEV genome. This probe annealed with DNA in the positions of 8.0- and 5.2-kb supercoiled DNA; we deduced that the latter represented the AEV genome. A representative sample of unintegrated AEV DNA is illustrated in Fig. 1, lane 2, where cDNA prepared from cloned AEV DNA (see below) was used to obtain an optimal signal.

To facilitate cloning of the covalently closed circular AEV DNA, it was desirable to separate the AEV genome from the large excess of helper virus DNA and from mitochondrial DNA. We therefore searched for restriction enzymes that would cleave only the contaminating DNAs, so that the supercoiled AEV DNA could be purified by extraction with phenol at pH 4.0. Our initial surveys were conducted with cDNA_{AEV} prepared by reverse transcription of viral RNA, but Fig. 1 illustrates definitive data obtained subsequently with cDNA obtained from cloned DNA (see below).

Hydrolysis with *Hpa*I cleaved the contaminating DNAs to linear forms (Fig. 1, lane 3), whereas the supercoiled AEV DNA had no *Hpa*I cleavage site (Fig. 1, lane 4). Other restriction endonucleases were also used to find an enzyme with a single recognition site in AEV DNA that would allow molecular cloning in a procaryotic vector. The enzymes *Xho*I and *Eco*RI appeared to have single cleavage sites in AEV DNA, as evidenced by the comigration of the AEV DNAs cleaved by these enzymes at the position expected for genome-length linear DNA (Fig. 1, lanes 5 and 6).

Figure 1 also illustrates that the AEV supercoiled DNA is more heterogeneous in size than the DNA of its helper. We attribute this finding to two factors: (i) the duplication of a sequence of 300 nucleotides in a fraction of the supercoils, as described for ASV (34) (such heterogeneity would affect the mobility of a 5.2-kb molecule more than that of an 8.5-kb molecule); or (ii) heterogeneity of the AEV genome in our uncloned virus stock (this conclusion will be substantiated below).

The AEV DNA was cloned in λ gtWES-B by the approach outlined above. In brief, purified supercoiled DNA was cleaved with *Hpa*I, and mitochondrial and helper virus DNAs were removed by extraction with phenol at pH 4.0. The circular AEV DNA was then cleaved with *Eco*RI and cloned into the RI site of λ gtWES-B. A total of 22 plaques with recombinant phage were chosen for further analysis; six of these recombinants annealed with AEV-specific probe. Four of these phage DNAs carried inserts of 5.0 to 5.5 kb; one insert was ca. 6.5 kb, and one was approximately 4.5 kb. We attribute the diversity of

FIG. 1. Characterization of supercoiled AEV DNA from infected cells. Circular DNA was prepared from QT-6 cells at 28 h after infection as described in the text. After digestion with restriction endonucleases and electrophoresis in a 1% agarose gel, the DNA was transferred onto a nitrocellulose sheet and annealed with cDNA_{rep} (lanes 1 and 3) or cDNA_{AEV} (lanes 2, 4–6). Lanes 1 and 2, undigested DNA; lanes 3 and 4, *Hpa*I-digested DNA; lane 5, *Eco*RI-digested DNA; lane 6, *Xho*I-digested DNA. Linear and supercoiled pBR313 and simian virus 40 DNA (9 and 5.2 kb, respectively) were used as size markers.

these inserts to the heterogeneity of the AEV DNA described above. We chose two recombinants for further study: AEV-10 and AEV-11, with inserts of 5.1 and 5.4 kb, respectively.

Physical map of the AEV genome. To establish a physical and genetic map of AEV DNA, we analyzed the cloned DNA by site-specific cleavage with restriction endonucleases. AEV DNA was recovered from the recombinant phage, purified, and digested with several enzymes. The fragments were separated by agarose gel electrophoresis, and their order on a linear map was determined by appropriate double digestions. The resulting map of AEV-10 is shown in Fig. 2. Fragment sizes indicated in the figure have been approximated to the nearest 100 base pairs. The following enzymes were found not to cleave the cloned AEV DNA: *Hind*III, *Hpa*I, *Kpn*I, *Pvu*I, *Sma*I, and *Xba*I. The two isolates studied, AEV-10 and AEV-11, have similar but not identical restriction maps. Restriction fragments derived from the leftward domain of AEV-11 were consistently ca. 300 base pairs longer than the corresponding fragments from AEV-10 (Fig. 3, lanes 1, 2, 8, and 9). This difference in size recalls the 300-base pair terminal redundancy that is found in the linear DNAs of other avian retroviruses and that occurs either once or twice in the circular forms of viral DNA (23). We therefore suspected that the redundant sequence (composed of nucleotide sequences from both the 5' and 3' termini of viral RNA) might be represented twice in the left-hand end of AEV-11 DNA and once in AEV-10 DNA. To pursue this possibility further and to determine the orientation of the molecules with

respect to the ASV genome, fragments generated by digesting AEV-10 and AEV-11 DNA with *Pst*I and *Ava*I were separated by gel electrophoresis, transferred to nitrocellulose sheets, and annealed with radioactive probes representing specific regions of the ASV genome. As shown in Fig. 3 (lanes 3 and 4), cDNA₃, annealed with the 1.0-kb *Pst*I fragment of AEV-10 and with the corresponding 1.3-kb fragment of AEV-11. These findings locate the redundant sequence within both fragments and are in accord with our explanation for the difference between the sizes of the two fragments. Analysis by annealing cDNA₃ to fragments generated by other enzymes has given similar results (Fig. 3, lane 10, and data not shown).

The *Pvu*II probe for *env* gave a hybridization pattern identical to that obtained with cDNA₃, (Fig. 3, lanes 7 and 13), suggesting that at least a portion of *env* is encoded in AEV and, as expected, is contiguous with the 3' terminal domain of the viral genome (5, 27). Sequences related to the *gag* gene of ASV were found in the 1.5-kb *Pst*I and the 0.3- and 0.8-kb *Ava*I fragments of AEV-10 (Fig. 3, lanes 6 and 12). These findings locate the *gag*-related part of the AEV genome between 1.5 and 2.5 kb from the left end of the cloned AEV DNA.

A cDNA representing the whole ASV genome annealed to the same fragments as the combined 3', *gag*, and *env* probes, as shown in Fig. 3, lanes 5 and 11. The finding that the ASV probes anneal to the 1.4-, 0.3-, and 0.8-kb *Ava*I fragments, but not to the 0.5-kb *Pst*I fragment, indicates that the sequences specific for AEV start between 2.1 and 2.5 kb from the left end and continue all the way to the right-hand end of the permuted cloned DNA (see Fig. 2). To prove this, the 2.5-kb *Pvu*II fragment allegedly carrying the AEV-specific sequences was purified by agarose gel electrophoresis, labeled *in vitro* with ³²P, and annealed with electrophoretically fractionated DNA from cells acutely infected with AEV and its helper virus. Despite the vast excess of helper virus DNA in the preparation (data not shown), the radioactive probe prepared with the 2.5-kb *Pvu*II fragment hybridized only with AEV DNA (Fig. 1, lanes 2, 4-6). The specificity of the AEV probe was further demonstrated by its exclusive annealing with AEV RNA that had been purified from virus and then subjected to gel electrophoresis and transfer to DBM paper (Fig. 4, lanes 1 and 4).

In conclusion, our data are consistent with the following order of sequences from left to right in the cloned AEV DNA: *env*-terminal redundancy (one or two copies)-*gag*-ASV-specific sequences. The data also show that at least 50% of

FIG. 2. Restriction endonuclease cleavage sites in cloned AEV-10 DNA. AEV DNA is depicted as it occurs in the recombinant phage; consequently, the topography of the AEV DNA is permuted with respect to the RNA genome of the virus. The estimated locations of the AEV-specific and ASV-related sequences are shown schematically at the bottom of the figure. TR, Terminal redundancy.

FIG. 3. *Pst*I and *Ava*I digests of AEV-10 and AEV-11 DNA. Cloned AEV DNA was purified, cut with restriction endonuclease *Pst*I (AEV-11, lanes 1 and 3; AEV-10, lanes 2 and 4-8) or *Ava*I (AEV-11, lane 8; AEV-10, lanes 9-13), and transferred to nitrocellulose sheets after agarose gel electrophoresis. DNA on filters was annealed with the following probes: *cDNA*_s, (lanes 3, 4, and 10); *cDNA*_{gag} (lanes 6 and 12); *cDNA*_{env} (lanes 7 and 13); *cDNA*_{rep} (lanes 5 and 11). Lanes 1, 2, 8, and 9 show ethidium bromide-stained DNA.

FIG. 4. Recovery of replicating AEV after transfection. Chicken cells were transfected with AEV DNA linked to RAV-1 DNA as described in the text. Cultures were serially passaged, and virus was harvested at 2-h intervals after the eighth passage. Viral RNA was purified, subjected to agarose gel electrophoresis, and transferred to activated DBM paper. RNA was annealed with *cDNA*_{REV} (lanes 1-3) or *cDNA*_{rep} (lanes 4-6). Lanes 1 and 4, viral RNA from cultures chronically infected with standard AEV; lanes 2 and 5, AEV-10 RNA; lanes 3 and 6, AEV-11 RNA.

the sequences are specific for AEV. Figure 2 illustrates the genetic composition and map of the AEV genome, based on our results and those of other workers (6, 26, 27).

Transfection with AEV DNA. Transfection of chicken fibroblasts with either the unintegrated or integrated form of ASV DNA apparently requires one round of viral replication and secondary infections before viral infection and cellular transformation can be established (8, 9).

As a result, transfection with the DNA of replication-defective retroviruses has proven to be difficult; we attribute this difficulty to the requirement that a single cell assimilate and express the DNA of both the helper virus and the defective transforming virus.

To show that AEV-10 and AEV-11 DNA contained nucleotide sequences that can lead to the transformation of chicken cells, a transfection assay was developed in which cloned DNA of RAV-1 was used to provide the functions of helper virus. Our procedure addressed two important considerations. (i) The cloned DNAs of both AEV and RAV-1 are permuted with respect to the linear order of the viral RNAs. We therefore found it advisable to ligate cloned viral DNAs into head-to-tail homodimers (or even higher orders of tandem arrays) before transfection, thereby creating representations of the full viral genome and increasing the efficiency of transfection (unpublished data). (ii) Successful transfection may require that a single cell assimilate both AEV and helper virus DNA. We augmented the chance that this would occur by joining the two viral DNAs with a neutral linker, a 3.8-kb DNA fragment derived from the *Eco*RI B fragment of λ gtWES-B by cleavage with *Sac*I (29). Since this fragment has an *Sac*I site at one end and an *Eco*RI site at the other, it serves conveniently to link the cloned AEV DNA (*Eco*RI termini) and the cloned RAV-1 DNA (*Sac*I termini). The ligations described by (i) and (ii) were carried out simultaneously, but (i) was favored by using the viral DNAs at fourfold-greater molar concentrations than the neutral linker.

Transfection with the ligand DNAs was car-

ried out by standard procedures. Cells were passaged repeatedly, and when the reverse transcriptase activity released to the culture medium paralleled the activities of cultures productively infected with helper virus, virus was harvested from the culture medium. No morphological transformation of the cultures was evident at this stage. RNA was extracted from purified virus, subjected to agarose gel electrophoresis, and transferred to activated DBM paper. As shown in Fig. 4, the preparation contained RNA that comigrated with marker AEV RNA and annealed with the AEV-specific probe described in the previous section.

We could envision two possible explanations for the failure of the transfected cultures to undergo morphological transformation: either AEV-10 and AEV-11 contain mutations that affect the ability of AEV to transform fibroblasts, or a large excess of helper virus interfered with the spread of AEV during the transfection. We pursued the latter possibility by two means. First, we infected secondary cultures of chicken embryo cells with a high multiplicity of the virus obtained from the transfection experiments. After three passages, the infected cells developed a morphology typical for fibroblasts transformed by AEV (Fig. 5). Virus recovered from these transformed cultures contained the genome of AEV (data not shown). Second, we used the virus generated by transfection to form individual foci or transformed cells under agar. The foci were then isolated and propagated en masse. Virus harvested from the resulting cultures transformed both fibroblasts (2×10^4 focus-forming units/ml) and bone marrow cells (5×10^2 focus-forming units/ml) in culture and caused both fibrosarcomas and erythroleukemia in chickens (our unpublished data). Figure 5E illustrates a typical focus of transformed bone marrow cells induced by the virus recovered from AEV-11.

We conclude that our protocol for transfection with cloned DNAs permits the recovery of infectious AEV in company with its helper virus. The ability of the recovered virus to transform fibroblasts and bone marrow cells, its tumorigenicity, the size of the viral RNA, and the presence of AEV-specific nucleotide sequences in the viral RNA all indicate that we have generated authentic AEV by transfection and that the viral genome was not appreciably disturbed by the procedures used to facilitate transfection.

DISCUSSION

Identity of the cloned DNA. We used molecular cloning to isolate and amplify the unintegrated DNA of AEV. All of our data indicate

that we have cloned the authentic AEV genome and that the genome has not been appreciably disturbed by the cloning procedure: (i) the length and topography of the cloned DNA conform to previous knowledge of the AEV genome (6, 27); (ii) radioactive cDNA prepared with a suitable restriction fragment from the cloned DNA reacted exclusively with the RNA and DNA of AEV; and (iii) transfection with the cloned DNAs in conjunction with the DNA of a helper virus gave rise to infectious virus with a genome and transforming potential characteristic of AEV.

Restriction map of cloned AEV DNA. We used site-specific cleavage with restriction endonucleases and hybridization with genetically defined cDNA's to prepare a linear map of the cloned AEV DNA. Our results agree with previous descriptions of the AEV genome obtained by analysis of heteroduplexes (27) and of T1 oligonucleotides (6). We detected *gag* sequences in AEV by using a *Bam*HI fragment (from cloned ASV DNA) that is specific for *gag* and represents over half of the gene (10). A radioactive probe prepared with this DNA fragment annealed to nucleotide sequences that extend from 1.4 kb to a point between 2.1 and 2.5 kb from the left end of the permuted AEV-10 DNA; these results suggest that the genome of AEV contains a minimum of 0.7 kb from the ASV *gag* gene. The maximum complexity of the *gag* sequences in AEV is more difficult to estimate because we have been unable to determine the leftward extent of the gene in the cloned AEV DNA. It is likely, however, that *gag* sequences extend to a point no farther than 1.0 kb from the left end of the cloned DNA, since all detected homology with the terminal redundancies of viral DNA lies to the left of that point.

The sequences related to *env* in AEV-10 were detected by their annealing with a 1.6-kb DNA fragment that represents the 3' domain of *env*. A fragment representing 750 nucleotides in the central portion of *env* did not anneal with AEV DNA (B. Vennström, unpublished data). These findings indicate that only a 3' terminal portion of *env* is encoded in the genome of AEV. We estimate that the maximum complexity of the *env* domain in AEV is ca. 0.4 kb; the domain is confined entirely to a 1.0-kb *Pst*II fragment from the left end of the cloned DNA, and 0.6 kb of this fragment can be accounted for by the 0.3-kb terminal redundancy and adjacent untranslated nucleotide sequences from the 3' domain of viral RNA (often denoted "c"; A. P. Czernilofsky, W. DeLorbe, R. Swanstrom, H. E. Varmus, J. M. Bishop, E. Tischer, and H. M. Goodman, submitted for publication). This conclusion conforms to previous estimates based on analysis of

FIG. 5. Transformation of chicken fibroblasts and bone marrow cells using virus recovered by transfection with cloned AEV DNA. Virus recovered after transfection was used to infect secondary cultures of chicken embryo cells. Photographs were taken at the fourth passage after infection. Medium was harvested from the cultures infected with virus generated from AEV-11 DNA and used to induce individual foci of transformed fibroblasts under agar. Foci were isolated and propagated *en masse*. Virus harvested from these cultures was tested for transforming activity in bone marrow cells (15). (A, B, and C) Fibroblasts infected with stock AEV, virus recovered from AEV-11 DNA, and virus recovered from AEV-10 DNA, respectively; (D) uninfected fibroblasts; (E) focus of bone marrow cells transformed by virus recovered from AEV-11 DNA.

oligonucleotides (6). By contrast, heteroduplex analysis failed to reveal any homology with *env* in the AEV genome (27); we attribute this discrepancy to instabilities in the heteroduplexes caused by mismatched nucleotides, a possibility acknowledged by Lai et al. (27).

The genome of AEV contains a specific domain that may be responsible for oncogenesis by the virus (6, 27). This domain is defined by the absence of homology with the genomes of other avian retroviruses, including avian leukosis, myelocytomatosis, myeloblastosis, and sarcoma

viruses. In accord with previous reports, we find that the alleged oncogene of AEV occupies at least 50% of the viral genome and is situated between the residual portions of *gag* and *env* (6, 27). Our restriction map of cloned AEV DNA provides a fairly precise localization of the AEV-specific domain: the junction with *gag* is bracketed by *Ava*I and *Pst*I sites located 2.1 to 2.5 kb, respectively, from the left end of the cloned DNA; the junction with *env* lies in the immediate vicinity of the *Eco*RI site.

The linear duplex DNA of retroviruses contains direct terminal redundancies (43). Although our data provide an approximate location of the redundant sequences in the permuted colony AEV DNA, we were unable to describe the redundancies in detail for want of suitable restriction sites within the sequences. Two lines of evidence indicate that the terminal redundancies of AEV, like those of other avian retroviruses (43), contain ca. 0.3 kb. First, the DNAs of AEV-10 and AEV-11 differ in length by 0.3 kb; we have attributed this difference to the presence of only one copy of the repeated sequence in AEV-10. Second, the unintegrated circular DNA of AEV occurs in two populations whose sizes differ by 0.3 kb. As expected from detailed studies on the circular DNAs of ASV (23), the additional DNA in the larger circles of AEV contains nucleotide sequences found in the terminal redundancy (unpublished data). We presume that the smaller circles have but one copy of the terminal redundancy, that the larger circles have two copies, and that the difference in size defines the mass of the reiterated sequence. These conclusions conform to previous findings for other retroviruses (43).

Transfection with cloned AEV DNA. We hope to use the cloned AEV DNA to generate new mutants of the virus by site-specific mutagenesis. It was, therefore, necessary that we develop a procedure that would permit the recovery of infectious virus from the cloned DNA by transfection; transformation of cells in the absence of viral replication would not suffice for our purposes. Since AEV has multiple defects in genes required for replication, we designed a procedure that would provide simultaneous transfection with a suitable helper virus. We initially hoped that cotransfection with unlinked DNAs would suffice (44), but this approach failed in our hands (unpublished data). We therefore joined AEV and helper virus DNA by means of a neutral linker fragment; the ligated DNAs gave rise to infectious AEV when transfected into chicken cells. We presume (but cannot prove) that the AEV and helper virus DNAs were independently transcribed into viral RNA

in response to promoter signals presumably carried by the viral DNA (34).

We tested the infectivity of AEV DNA carrying either one or two copies of the terminal redundancy and recovered infectious AEV in both instances. These findings suggest that the frequency of the redundant sequence is of no consequence to the infectivity of the DNA, in accord with a previous report for the cloned DNA of murine sarcoma virus (18). It remains possible, however, that recombination between AEV and helper virus during transfection rescued AEV DNA that might otherwise not be infectious.

Utility of cloned DNA. The genetic and biochemical analyses of AEV are both impeded by properties of the virus. Our work illustrates how these impediments can be circumvented by the use of molecular cloning: the viral genome can be isolated and propagated without the encumbrance of a helper virus; the genome can be rigorously identified by means of infectivity tests, a procedure that is not possible with the isolated RNA of retroviruses; viral DNA can be prepared in large quantities for structural analyses; restriction fragments from the cloned DNA can be used to prepare cDNA's whose genetic composition is well defined; and site-specific mutagenesis performed on the cloned DNA can be used to define and dissect genetic domains within the genome. We expect to exploit these advantages in our efforts to explore the mechanism of oncogenesis by AEV and the phylogenetic origins of the viral oncogene (37).

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