Organization of Chicken DNA Sequences Homologous to the Transforming Gene of Avian Myeloblastosis Virus

II. Isolation and Characterization of λ *proto-amv* DNA Recombinant Clones from a Library of Leukemic Chicken DNA

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Several λ *proto-amv* recombinants isolated from a λ Charon 4A library of leukemic chicken DNA were analyzed by using various restriction endonucleases and hybridization with specific probes representing different regions of the transforming gene of avian myeloblastosis virus. The position of 30 sites for 11 different restriction endonucleases was established in the *proto-amv* region of chicken DNA. Identical restriction endonuclease maps were obtained for the normal and leukemic DNAs in the *proto-amv* domain, which covers 8 to 9 kilobases of DNA. The cellular genetic elements homologous to the cellular sequence (*amv*) inserted into the avian myeloblastosis virus genome are contained within six major *proto-amv* segments which are interrupted by at least five large DNA regions lacking homology with *amv*.

The structural organization, expression, and evolutionary conservation of cellular DNA sequences (protooncogenes) homologous to the oncogenes of several avian, feline, murine, and simian retroviruses suggest that these protooncogenes represent essential normal eucaryotic genes (2-4, 8, 11, 13, 22, 25, 29-31, 33). All of the known cellular protooncogenes, except the protooncogene of Moloney sarcoma virus (18) and *c-ras-I* of Harvey sarcoma virus (11), are interrupted by intervening sequences (11, 14-16, 19, 24, 34). In the avian myeloblastosis virus (AMV) genome there is a cellular insertion (*amv*) 1,200 bases long which has been substituted for most of the envelope gene to generate the viral oncogene (29). In our previous studies (19, 20), we established that the cellular DNA sequences (*proto-amv*) homologous to *amv* are present within a unique chicken DNA segment 8 to 11 kilobases (kb) long containing a minimum of four large introns, which were identified by mapping of restriction endonuclease sites in total normal chicken DNA. This kind of analysis was limited by the number of restriction endonucleases used, the distribution of the DNA restriction sites, and the short length of the *proto-amv* segment in some DNA fragments. Comparisons of the *proto-amv* fragments gener-

ated by restriction enzyme digestion of the total DNAs of normal and leukemic cells revealed that there was no apparent reorganization of the normal *proto-amv* region as a result of leukemogenesis (20). Therefore, we could take advantage of our λ Charon 4A library of leukemic chicken DNA partially digested with *EcoRI* (28) to isolate λ *proto-amv* recombinants and better characterize the organization of the cellular *proto-amv* region by using amplification of *proto-amv* fragments in λ recombinant clones and the presence of certain restriction endonuclease sites in this λ vector (12).

The restriction endonuclease analysis described here confirmed the general organization of the *proto-amv* sequences in chicken DNA described previously but also revealed an additional intron which was not detected in the analysis of total cellular DNA (20). With six different enzymes 16 restriction sites were found to be identical in normal and leukemic chicken DNAs. The use of five additional restriction endonucleases and subcloning in pBR322 of some *proto-amv* fragments allowed us to determine more precisely the overall organization of the *proto-amv* domain in chicken DNA.

MATERIALS AND METHODS

Cloning. In all transformation experiments *Escherichia coli* HB101 (7) was the recipient and pBR322 was the vector (6). The transformation procedure has been described previously (19). The recombinants

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were screened as reported previously (20). Colony hybridization was performed as described by Grunstein and Hogness (17).

Preparation of λ phage lysates and purification of virions. Single plaques were suspended in 100 μ l of λ synthetic medium (20 mM Tris-hydrochloride, pH 7.5, 100 mM NaCl, 1 mM $MgSO_4$, 0.01% gelatin) poured onto agar overlays (0.8% agar in TYE medium [10 g of tryptone per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter]) containing *E. coli* DP50 Sup F (5) and incubated at 37°C overnight. The phage harvested from the lysis zone by low-speed centrifugation (4,000 rpm for 10 min in a Beckman JS2 rotor) was used to prepare a primary stock on TYE agar plates, which was used to infect strain DP50 Sup F (multiplicity of infection, 0.1) for the preparation of liquid stocks (in TYE medium containing 10 mM $MgCl_2$). When lysis was complete (10 h for 2×10^8 cells initially infected), the phage was precipitated by adding 35 g of NaCl per liter and 80 g of polyethylene glycol 6000 (Sigma Chemical Co., St. Louis, Mo.) per liter at 4°C overnight. A titer of about 8×10^{11} to 9×10^{11} PFU/ml was usually obtained. After 1 h of incubation in the presence of DNase (200 μ g/ml), the resuspended precipitate was treated once with 1 volume of chloroform, and the phage particles were purified by CsCl density equilibrium centrifugation in a Beckman SW40 rotor at 35,000 rpm for 40 h (4°C).

DNA purification. Plasmid DNA was purified as described by Curtiss et al. (10) after amplification in the presence of chloramphenicol (9). The plasmid DNA was banded twice in a cesium chloride gradient when it was to be used for nick translation.

λ hybrid phage DNA was extracted from purified virions, dialyzed against 10 mM Tris-hydrochloride (pH 7.5)–10 mM $MgSO_4$, and mixed with 0.1 volume of $10\times$ buffer C (500 mM Tris-hydrochloride, pH 7.4, 100 mM NaCl, 100 mM EDTA) containing sodium dodecyl sulfate (final concentration, 0.2%). After 250 μ g of proteinase K per ml was added and 1 h of incubation at 37°C, the λ DNA was extracted once with 1 volume of a 1:1 mixture of phenol and chloroform-isoamyl alcohol (chloroform-isoamyl alcohol, 24:1) and twice with 1 volume of chloroform-isoamyl alcohol. The λ DNA was precipitated after NaCl (0.15 M) and 3 volumes of cold absolute ethanol were added, spooled out, dried under a vacuum, and dissolved in 0.5 to 1 ml of sterile distilled water. About 300 to 400 μ g of DNA was obtained per liter of initial lysate.

Nick translation of DNA. Total purified plasmid DNA or electroeluted DNA fragments were nick translated (21) as previously described (19) in the presence of 100 μ Ci of [α - 32 P]dCTP (New England Nuclear Corp., Boston, Mass.). The buffer used contained 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.8; Calbiochem, La Jolla, Calif.), 5 mM $MgCl_2$, 1 mM dithiothreitol, and 500 μ g of pentox bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.) per ml.

Preparation and hybridization of DNA blots. DNA was enzyme treated and electrophoresed in agarose gels as described previously (19). The DNA fragments were blotted onto nitrocellulose membrane filters (Millipore Corp., Bedford, Mass., or Schleicher & Schuell Co., Keene, N.H.) as described by Southern (26). Hybridizations were performed as described previously (19).

Derivation of specific *amv* and *proto-amv* probes. Purified DNA from the λ -AMV provirus recombinant 11A1-1 (28) was digested with endonuclease *Hind*III, and the resulting fragments were separated in a 0.8% agarose gel. The AMV 5'-proximal 2.9-kb *Hind*III fragment was electroeluted, extracted as described previously (19), and cloned in pBR322 previously digested with *Hind*III under conditions described previously (19). Screening of the colonies was performed as described previously (20). One of the clones (clone HI91) containing the *Hind*III fragment (Fig. 1) was further analyzed to determine the orientation of the insertion with respect to pBR322 DNA. *Eco*RI and *Bam*HI digests of pBR322/HI91 DNA revealed that the 5' end of the insertion is located toward the *Bam*HI site of pBR322.

The isolation and characterization of *amv* recombinant clones pBR322/KX162, pBR322/HAX₄, and pBR322/SES3 have been described previously (19, 20).

proto-amv recombinant subclones pBR322/P542 and pBR322/P873 were prepared from λ -*proto-amv* recombinants 1041 and 111, which contain 5.4- and 8.7-kb *Eco*RI fragments, representing the 5' and 3' ends of the *proto-amv* sequences, respectively (19). The DNA

FIG. 1. Derivation of the probes representing specific regions of *amv* and *proto-amv* subcloned in pBR322. Clone HI91 represents the 2.9-kb AMV 5'-proximal *Hind*III fragment. Clones HAX₄ and SES3 have been described previously (19, 20). Clones P873 and P542 correspond to the 3'- and 5'-terminal regions of the *proto-amv* sequences, respectively (19). LTR, Long terminal repeat.

was digested with *Eco*RI and electrophoresed in a 0.8% agarose gel. The 5.4-kb band from λ 1041 and the 8.7-kb band from λ 111 were electroeluted and purified as previously described (19) before cloning in pBR322 at the *Eco*RI site. The recombinants were screened by colony hybridization with 32 P-labeled HAX₄ DNA, as reported previously (20). The orientation of the 5.4-kb fragment in pBR322/P542 was established from a *Hind*III digest of the recombinant DNA. The 5' end of the insertion is located toward the *Pst*I site of pBR322. A *Bam*HI digest of pBR322/P873 showed that the 8.7-kb fragment is inserted with its 5' end toward the *Bam*HI site of pBR322.

Isolation of λ proto-amv recombinant clones. Chicken λ DNA recombinants 111, 233, 533, 1041, and 1132 were selected on the basis of positive hybridization with the *Kpn*I-*Xba*I AMV probe KX162 and lack of hybridization with the 3.9-kb internal *Eco*RI fragment isolated from myeloblastosis-associated virus (19).

Combined use of sub-amv probes SES3, HI91, and HAX₄ permitted the isolation of chicken- λ recombinants containing a proto-amv region (2.0-kb *Eco*RI fragment) not represented in the clones obtained previously (19). A total of 1.5×10^6 plaques from the λ Charon 4A library of leukemic chicken DNA partially digested with *Eco*RI (28) were screened by using the in situ plaque hybridization technique of Benton and Davis (1). Duplicate nitrocellulose filters were hybridized either to the HI91 probe to detect clones containing viral sequences or to the amv-specific SES3 probe. A total of 38 plaques hybridized with the SES3 probe, and 13 also hybridized with the HI91 probe. These 13 plaques were discarded since they probably represented viral sequences. The remaining 25 plaques were replated once and hybridized with either the SES3 probe or the P873 probe. One plaque which hybridized with only the SES3 probe (plaque 121) and one plaque

which hybridized with both probes (plaque 322112) were picked, replated twice, and checked for hybridization with both HAX₄ and SES3. High-titer stocks were used to obtain purified recombinant DNA from λ 121 and λ 322112.

Physical and biological containment. This work was carried out at P2EK2 containment levels according to the current guidelines of the National Institutes of Health.

RESULTS

***Eco*RI or *Hind*III analysis of λ proto-amv DNA recombinant clones 111, 233, 533, 931, 1041, 1132, and 1321.** Eight different λ proto-amv recombinant clones were selected and purified from a λ Charon 4A library of leukemic DNA partially digested with *Eco*RI on the basis of positive hybridization with AMV probe KX162 and lack of hybridization with the internal 4-kb *Eco*RI fragment of myeloblastosis-associated virus (19). These λ recombinant clones were amplified, and their DNAs were purified as described above. After digestion with *Eco*RI or *Hind*III, the DNA fragments were separated by electrophoresis in 0.8% agarose gels, blotted onto nitrocellulose filters, and hybridized to 32 P-labeled DNA from the HAX₄ probe.

*Eco*RI digestion of λ clones 533, 931, 1132, and 1321 revealed a single 8.7-kb fragment having homology to amv (Fig. 2A). *Eco*RI-digested clones 111 and 233 contained two amv homologous fragments (8.7 and 2.1 kb), whereas *Eco*RI-digested λ 1041 DNA contained a 5.4-kb frag-

FIG. 2. *Eco*RI and *Hind*III restriction maps of λ -chicken DNA recombinant clones. DNAs (0.5 μ g) from λ recombinant clones 111, 233, 533, 931, 1041, 1132, and 1321 were digested with either *Eco*RI (A) or *Hind*III (B). After electrophoresis in 0.8% agarose gels, the gels were stained with ethidium bromide, photographed, blotted onto nitrocellulose, hybridized to the 32 P-labeled HAX₄ probe, and autoradiographed. *Hind*III-digested λ DNA fragments served as size markers. Parts of the λ arms generated by *Eco*RI digestion are represented by cross-hatched bars, the longest one representing the long arm and the shortest one representing the short arm. The arrows indicate *Eco*RI sites (A) and *Hind*III sites (B). The solid boxes represent proto-amv segments.

ment. The sizes of the *EcoRI* fragments detected in these digests were similar to the sizes of the fragments detected by HAX₄ hybridization to an *EcoRI* digest of total C/O chicken DNA (20). This indicates that the cloned segments from leukemic DNA probably represent *proto-amv* sequences previously mapped in normal chicken DNA (20).

The presence of a *HindIII* site on the short arm of λ Charon 4A 5.1 kb from the *EcoRI* site (12) was used to orient the chicken DNA insertion with respect to the *EcoRI*-generated arms of the λ vector. After *HindIII* digestion and hybridization to HAX₄, a single *proto-amv* band 9.5 kb long was detected in λ clones 931, 1132, and 1321, positioning a *HindIII* site within the chicken DNA insertion at 4.4 kb from the λ short arm (Fig. 2B). Hybridization of *HindIII*-digested λ clones 533 with HAX₄ revealed a 20-kb band, indicating that the chicken DNA insertion in λ clone 533 is oriented in the opposite direction of the insertions in λ clones 931, 1132, and 1321. Thus, λ clones 931, 1132, and 1321 carry the same set of *proto-amv* sequences (8.7-kb *EcoRI* fragment) which are also present in λ clone 533 but in the opposite orientation.

λ clones 111 and 233 showed similar restriction patterns after ethidium bromide staining of their *EcoRI* or *HindIII* digests because of the presence of a *HindIII* site 1.3 kb from either end of the chicken DNA insertion (Fig. 2B). Hybridization of the *EcoRI* digests of these clones with HAX₄ also revealed identical *proto-amv* band patterns. However, hybridization of *HindIII*-digested λ 111 with HAX₄ revealed bands at 5.2 and 6.4 kb, whereas hybridization of *HindIII*-digested λ 233 produced 5.2- and 20-kb bands. Since the *HindIII* 5.2-kb *proto-amv* band was detected in both clones, this band corresponds to an internal fragment. The 6.4-kb *proto-amv* fragment detected in λ 111 resulted from the presence of a *HindIII* site within the chicken DNA that is 1.3 kb from the λ short arm. Therefore, λ 111 and λ 233 carry the same set of *proto-amv* sequences (*EcoRI* 8.7- and 2.1-kb fragments), but these sequences are in the opposite orientation with respect to the λ clone Charon 4A arms.

Hybridization of the *HindIII* digest of clone λ 1041 with HAX₄ revealed that the *proto-amv* sequences are located near the long arm of λ Charon 4A in a long 20-kb fragment.

Analyses of the fragments detected after ethidium bromide staining and after hybridization with HAX₄ in *EcoRI* or *HindIII* digests and in *EcoRI-HindIII* double digests (Fig. 3) led to construction of the restriction maps shown in Fig. 2 (20).

λ recombinant clones 111, 233, 533, 1041, and 1132 were chosen for further study.

FIG. 3. *HindIII-EcoRI* double digestion of λ recombinant clones. DNAs (0.5 μ g) from λ recombinants were digested with *HindIII* and *EcoRI*. The blots prepared after electrophoresis in a 0.8% agarose gel were hybridized to HAX₄. Ethidium bromide staining was used for lanes a through d (lane a λ 111; lane b, λ 1041; lane c, λ 533; lane d, λ 1132); the corresponding blots are shown in lanes a' through d', respectively. *HindIII*-digested λ DNA was run as size markers.

Restriction endonuclease analysis of λ *proto-amv* recombinant clones 111, 233, 533, 1041, and 1132. (i) *HindIII-EcoRI* double digestion. Ethidium bromide staining of an *EcoRI-HindIII* double digest of λ 533 and λ 1132 revealed four bands (4.4, 2.8, 2.5, and 1.2 kb) in addition to the λ Charon 4A-specific fragments (Fig. 3). Hybridization with HAX₄ detected the 4.4-kb band, which, therefore, corresponds to the *proto-amv*-containing fragment located at the 5' terminus of the chicken DNA insertion in λ clones 533 and 1132.

Ethidium bromide staining of an *EcoRI-HindIII* double digest of λ 111 revealed three bands (4.4, 2.8, and 1.2 kb) in addition to the λ fragments (Fig. 3). Hybridization with HAX₄ detected two bands (4.4 and 1.2 kb). The 4.4-kb *proto-amv* fragment was the same fragment present in λ 533 and λ 1132. In λ 111 the 1.2-kb *proto-amv* fragment probably originated from the 2.1-kb *EcoRI proto-amv* fragment (20), locating the *HindIII* site 1.2 kb from one of the *EcoRI* sites.

Hybridization with HAX₄ after *EcoRI-HindIII* double digestion of λ 1041 revealed a single 2.4-kb fragment. This placed a *HindIII* site 2.4 kb from the 3' terminus of the 5.4-kb *proto-amv EcoRI* fragment, which constitutes the 5' terminus of the *proto-amv* domain (20).

(ii) *KpnI* or *BamHI* digestion. Endonuclease

*Kpn*I cleaves λ Charon 4A DNA 0.9 kb from the long-arm *Eco*RI site and 7.35 kb from the short-arm *Eco*RI site (Fig. 4). *Bam*HI cleaves λ Charon 4A DNA 14 kb from the long-arm *Eco*RI site and 4.9 kb from the short-arm *Eco*RI site (12).

*Kpn*I digestion of λ clones 111, 233, 533, and 1132 indicated that the chicken DNA insertion of these clones did not contain a *Kpn*I site, and hybridization with HAX₄ detected only a large fragment (15 to 17 kb) (Fig. 4A). *Kpn*I digestion of λ 1041 revealed several *Kpn*I sites within the chicken DNA insertion, and hybridization with HAX₄ detected a single *proto-amv* fragment of 3.7 kb (Fig. 4A, lane d'). This indicates that the

proto-amv sequences in λ 1041 are adjacent to the λ long arm.

When a *Bam*HI digest of λ 111 DNA was hybridized to HAX₄, two bands (6.9 and 5.4 kb) were detected (Fig. 4B). *Bam*HI digestion of λ 233 and hybridization to HAX₄ (data not shown) revealed the 5.4-kb fragment and a large 16-kb fragment. Therefore, in λ 111 the 6.9-kb *Bam*HI fragment includes part of the short arm of λ Charon 4A and positions a *Bam*HI site within the chicken DNA insertion 2.0 kb from the 5' end. The 5.4-kb *Bam*HI *proto-amv* fragment is adjacent to the 6.9-kb fragment in the insertion.

As expected from the *Hind*III and *Eco*RI

FIG. 4. *Kpn*I or *Bam*HI digestion of λ recombinant clones. DNAs (0.5 μ g) from λ recombinants were digested with either *Kpn*I (A) or *Bam*HI (B). The blots prepared after electrophoresis in 0.8% agarose gels were hybridized to HAX₄. Ethidium bromide staining was used for lanes a through e (lane a, λ 233; lane b, λ 1132; lane c, λ 533; lane d, λ 1041; lane e, λ 111); the corresponding blots are shown in lanes a' through e', respectively. *Hind*III-digested λ DNA fragments were used as size markers. In the insertions the arrows indicate recognition sites for *Kpn*I (K) and *Bam*HI (B).

mapping, hybridization of λ 533 digested with *Bam*HI with HAX₄ revealed a large fragment (19 kb) which included part of the λ long arm (Fig. 4B). Hybridization of *Bam*HI-digested λ 1132 with HAX₄ detected a 10-kb fragment, indicating that a *Bam*HI site is present 5.1 kb from the 5'-terminal *Eco*RI site of the chicken DNA insertion. Ethidium bromide staining detected a 5.9-kb *Bam*HI fragment common to λ 111, λ 233, and λ 1132 (Fig. 4B). An additional *Bam*HI fragment of 2.9 kb which did not hybridize to HAX₄ was present in λ 533 and λ 1132, presumably near the 3' end of the chicken DNA insertion.

A long fragment (24 kb or longer) containing

part of the λ long arm was detected in the *Bam*HI digest of λ 1041 after hybridization with HAX₄ (Fig. 4B).

(iii) *Hind*III-*Bam*HI double digestion. Hybridization with HAX₄ of λ 111 and λ 233 successively digested with *Hind*III and *Bam*HI revealed common fragments 4.6 and 0.85 kb long plus a 6.0-kb fragment in λ 111 and a 15-kb fragment in λ 233 (Fig. 5). Thus, the 4.6- and 0.85-kb fragments are internal. The 6.0-kb *proto-amv* fragment detected in λ 111 corresponds to the fragment delineated by the *Hind*III site in chicken DNA that is 1.2 kb from the 5'-terminal *Eco*RI site and by the *Bam*HI site in the λ short arm that is 4.9 kb from the same *Eco*RI site. In λ

FIG. 5. *Bam*HI-*Hind*III double digestion of λ recombinant clones. DNAs (0.5 μ g) from λ recombinants were digested with *Bam*HI and *Hind*III, electrophoresed in 0.8% agarose gels, and hybridized to HAX₄. Ethidium bromide staining was used for lanes a through e (lane a, λ 233; lane b, λ 1132; lane c, λ 533; lane d, λ 1041; lane e, λ 111); the corresponding blots are shown in lanes a' through e', respectively. *Hind*III-digested λ DNA was run as size markers. In the insertions the arrows indicate recognition sites for *Hind*III (H) and *Bam*HI (B).

233, which contains the insertion in the opposite orientation the 15-kb *proto-amv*-containing fragment is delineated by the *Hind*III site that is 1.2 kb into chicken DNA and by the *Bam*HI site that is 14 kb from the *Eco*RI site of the λ long arm. The faint 0.85-kb band (Fig. 5, lanes a' and e') detected in λ 111 and λ 233 must have resulted from cleavage of the *Hind*III and *Bam*HI sites that are located 1.2 and 2.0 kb, respectively, from the 5' terminus of the chicken DNA. Since the viral insertion (*amv*) does not contain a *Hind*III or *Bam*HI site, hybridization of this fragment with HAX₄ indicates that there is another nonhomologous intervening sequence between the *proto-amv* sequences located between the 5' terminus and the 5'-proximal *Bam*HI site of the chicken DNA. The relative intensities of the bands indicate that the *proto-amv* sequence is short in this 0.85-kb *Hind*III-*Bam*HI fragment. For this reason this intervening sequence was not detected in our previous study in which we used total chicken DNA (20).

As established above (Fig. 2B), there is a *Hind*III site 4.4 kb from the 5'-terminal *Eco*RI site of the chicken DNA in λ 533 and λ 1132. After double digestion with *Hind*III and *Bam*HI, this site yielded a 9.3-kb *proto-amv* fragment in λ 1132 and a large fragment (approximately 19 kb) in λ 533 (Fig. 5).

Only one large fragment (approximately 16 kb) was detected after hybridization with HAX₄ of λ 1041 DNA doubly digested with *Hind*III and *Bam*HI. This confirms that there is a *Hind*III site 2.4 kb from the 3' end of the chicken DNA insertion.

(iv) ***Kpn*I-*Eco*RI, *Kpn*I-*Hind*III, or *Kpn*I-*Bam*HI double digestion.** Double digestion of the λ recombinant clones with *Eco*RI and *Kpn*I or *Hind*III and *Kpn*I confirmed that only the DNA insertion of λ 1041 contains a *Kpn*I site (data not shown). After hybridization with HAX₄, the band pattern detected in the *Kpn*I-*Eco*RI double digests of λ 111, λ 533, and λ 1132 was identical to the pattern detected in single *Eco*RI digests. Hybridization with HAX₄ of the *Kpn*I-*Eco*RI double digest of clone λ 1041 revealed a 2.6-kb fragment.

Hybridization with HAX₄ of the *Hind*III-*Kpn*I digest of λ 111 showed the same band pattern as the pattern that was obtained with *Hind*III alone. Hybridization of the *Kpn*I-*Hind*III double digest of λ 233 detected a 2.1-kb band caused by cleavage of the *Kpn*I site located in the λ long arm at 0.9 kb from the *Eco*RI site. The same *Kpn*I site within λ also accounted for a 5.3-kb band detected after hybridization of the *Kpn*I-*Hind*III double digest from clone λ 533. Cleavage of the single *Kpn*I site in the small arm of λ gave rise to a 10-kb fragment which hybridized to HAX₄ in a *Hind*III-*Kpn*I double digest of λ

1132. This confirmed the presence of a *Hind*III site 4.4 kb from the 5'-terminal *Eco*RI site in the insertion of λ 1132.

The 3.4-kb fragment detected in the *Kpn*I-*Hind*III double digest of λ 1041 after hybridization to HAX₄ (data not shown) and the 2.4-kb fragment detected earlier in the *Hind*III-*Eco*RI double digest confirmed that the *proto-amv* sequences are adjacent to the long arm of λ in λ 1041 and established the relative positions of the *Kpn*I, *Hind*III, and *Eco*RI sites.

*Kpn*I-*Bam*HI double digestion of the λ recombinant clones (data not shown) confirmed the positions of the *Kpn*I and *Bam*HI sites determined by single enzyme digestions (Fig. 4).

(v) ***Xba*I-*Bam*HI double digestion, *Xba*I-*Hind*III double digestion, or *Xba*I single digestion.** There is no *Xba*I site in λ Charon 4A DNA. Two *proto-amv* fragments (6.8 and 3.4 kb) were detected in the *Xba*I-*Bam*HI double digest of λ 111, and two *proto-amv* fragments (>20 and 3.4 kb) were detected in the *Xba*I-*Bam*HI double digest of λ 233 (Fig. 6A). This indicates that the 3.4-kb fragment is internal. The position of the *Bam*HI sites in λ Charon 4A suggests that the 6.8-kb fragment in λ 111 resulted from *Bam*HI digestion alone.

Digestion of λ 111 with *Xba*I alone produced two *proto-amv* bands (7.0 and 17 kb), whereas a similar digestion of λ 233 produced two *proto-amv* bands (7.0 and >20 kb) (Fig. 6C). Thus, the 7.0-kb fragment results from the presence of two *Xba*I sites encompassing *proto-amv* sequences within the chicken DNA insertion. This finding positions one *Xba*I site on the 5' side of the 3'-proximal *proto-amv* segment 3.4 kb upstream from the *Bam*HI site located in the *Eco*RI 8.7-kb fragment. The 3.4-kb *proto-amv* fragment was also detected in λ 533 and λ 1132 digested with *Xba*I and *Bam*HI. A long juncture fragment (>20 kb) was detected by using HAX₄ in λ 1041 DNA digested with *Xba*I and *Bam*HI.

Digestion with *Xba*I and *Hind*III generated two *proto-amv* bands in λ 111 (6.3 and 2.6 kb) (Fig. 6B). Two bands (>20 and 2.6 kb) were also detected in λ 233. The 2.6-kb *proto-amv* band in λ 111 and λ 233 presumably represents two *proto-amv* fragments of approximately the same size (Fig. 5 and 6). Clones λ 533 and λ 1132 digested with *Hind*III and *Xba*I contained a 2.6-kb *proto-amv* fragment. This confirms the position of the *Xba*I site at 1.8 kb downstream from the 5'-terminal *Eco*RI site of the 8.7-kb *proto-amv Eco*RI fragment. Digestion of λ 1041 with *Xba*I and *Hind*III generated one long *proto-amv* fragment (>20 kb), indicating that there is no *Xba*I site within the 2.4-kb *proto-amv* fragment delineated by the *Hind*III and 3'-terminal *Eco*RI sites in this recombinant clone.

(vi) ***Sma*I-*Eco*RI double digestion or *Sal*I single**

digestion. The positions of the *Sma*I sites in λ Charon 4A are not known. In the viral *amv* insertion the single *Sma*I site is located 150 base pairs downstream from the single *Eco*RI site (19, 23). *Sma*I sites have been mapped previously in normal chicken DNA at 0.5 kb from the 5' end and within the 5'-proximal 5.4-kb *Eco*RI *proto-amv* fragment and at 1.1 kb outside the 3' end of the same fragment (20).

Double digestion of λ 1041 with *Sma*I and *Eco*RI generated a 5.0-kb *proto-amv* band (data not shown), confirming the presence of the 5'-proximal *proto-amv Eco*RI fragment in λ 1041 and the presence of a *Sma*I site 0.5 kb from its 5' end. Double digestion of λ 111, λ 233, λ 533, and λ 1132 with *Sma*I and *Eco*RI and hybridization with HAX₄ revealed the same *proto-amv* band patterns as those generated by digestion with *Eco*RI alone. This indicates the absence of a

*Sma*I site in the *proto-amv Eco*RI fragments of all of these recombinant clones.

*Sal*I digestion was performed on all of our available *proto-amv*-containing λ recombinant clones, and the resulting blots were hybridized to the HAX₄ probe. The results (data not shown) revealed that none of the chicken DNA insertions contained a *Sal*I site. A large (>20-kb) *proto-amv* band was detected in all of the *Sal*I digests. This is in agreement with the conclusion drawn from the analysis of the *Sma*I-*Eco*RI double digests. In addition, none of the *Sal*I digests hybridized with the SES3 *amv* probe (data not shown), which contains only *amv* sequences between the *Eco*RI and *Sal*I sites (including the *Sma*I site). This observation revealed that the λ *proto-amv* recombinant clones described above did not represent the *proto-amv* sequences in their entirety as they were previ-

FIG. 6. *Xba*I single digestion, *Xba*I-*Bam*HI double digestion, or *Xba*I-*Hind*III double digestion of λ recombinant clones. DNAs (0.5 μ g) from λ recombinants were digested with either *Xba*I and *Bam*HI (A), *Xba*I and *Hind*III (B), or *Xba*I alone (C). The blots obtained after electrophoresis in 0.8% agarose gels were hybridized with HAX₄. Ethidium bromide staining was used for lanes a through e (lane a λ 233; lane b, λ 1132; lane c, λ 533; lane d, λ 1041; lane e, λ 111); the corresponding blots are shown in lanes a' through e', respectively. *Hind*III-digested λ DNA was run as size markers. In the insertions the arrows indicate recognition sites for *Bam*HI (B) and *Xba*I (X).

FIG. 7. Digestion of λ 322112 with *Hind*III, *Eco*RI, *Sal*I, *Sma*I, or *Bst*EII. DNA (0.5 μ g) from λ 322112 was digested with either *Hind*III (A, B, and C, lane a), *Eco*RI (A, B, and C, lane b), *Sal*I (D, E, and F, lane a), *Sma*I (D, E, and F, lane b), or *Bst*EII (D, E, and F, lane c). After electrophoresis in 0.8% agarose gels and staining with ethidium bromide (A and D), the blots were hybridized to the SES3 probe (B and E), washed carefully, and rehybridized with the HAX₄ probe (C and F). A mixture of *Hind*III-digested λ DNA and *Hae*III-digested ϕ X174 RF DNA was run as size markers. Bs, *Bst*EII; E, *Eco*RI; Sm, *Sma*I; Sa, *Sal*I; H, *Hind*III.

ously mapped within total chicken DNA (20). Therefore, other *proto-amv*-containing λ recombinant clones were isolated, and two clones, λ 322112 and λ 121, were chosen for further analysis.

Restriction endonuclease analysis of λ *proto-amv* DNA recombinant clone 322112. (i) *Eco*RI or *Hind*III digestion. Ethidium bromide staining of λ 322112 DNA digested with *Eco*RI revealed three bands (8.7, 2.0, and 0.95 kb) in addition to the two λ Charon 4A arms (Fig. 7A). (The 0.95-kb band is not visible in Fig. 7A.) In a *Hind*III digest of the same DNA, four bands ($\geq$ 21, 8.0, 5.2, and 1.25 kb) appeared in addition to the 5.7-kb band typical for λ Charon 4A DNA (Fig. 7A).

Hybridization of the *Eco*RI or *Hind*III digest with the SES3 probe detected a 2.0-kb *Eco*RI fragment and a $\geq$ 21-kb *Hind*III fragment (Fig. 7B), whereas hybridization with the HAX₄ probe detected three *Eco*RI fragments (8.7, 2.0, and 0.95 kb) and three *Hind*III fragments ($\geq$ 21, 5.2, and 1.25 kb) (Fig. 7C). The 2.0-kb *Eco*RI band detected with the HAX₄ probe was broad, as it corresponds to the two adjacent *proto-amv* *Eco*RI fragments of 2.0 and 2.1 kb (20). The 0.95-kb band is faint because it contains a very short stretch of *proto-amv* sequences (20). The

sizes of the *proto-amv* fragments that hybridized with the two probes are in agreement with the sizes obtained with total C/O chicken DNA (20). Therefore, it appears that the λ 322112 clone contains the two *proto-amv* *Eco*RI fragments (8.7 and 2.1 kb) carried by λ 111 plus the *proto-amv* 0.95-kb *Eco*RI fragment and the *proto-amv* 2.0-kb *Eco*RI fragment which hybridizes specifically with the SES3 probe (20). The orientation of the chicken DNA insertion in λ 322112 with respect to the λ Charon 4A arms was determined from the *Hind*III 21-kb fragment which hybridized with both the SES3 probe and the HAX₄ probe. This positioned the 0.95-kb *Eco*RI fragment adjacent to the long arm of λ Charon 4A. These findings are summarized in Fig. 7.

(ii) *Bst*EII, *Sma*I, or *Sal*I digestion. λ Charon 4A DNA does not contain a *Sal*I site, and the location of the *Bst*EII and *Sma*I sites have not been determined. In *amv* a *Bst*EII site is located 34 base pairs downstream from the single *Eco*RI site (23). To investigate whether this *Bst*EII site exists at the same position in the 2.0-kb *Eco*RI *proto-amv* fragment which hybridizes with the SES3 probe, λ 322112 DNA was digested with *Bst*EII and hybridized to the SES3 and HAX₄ probes.

Ethidium bromide staining of λ 322112 DNA digested with *Bst*EII revealed 13 fragments (Fig. 7D). Hybridization with the SES3 probe (Fig. 7E) detected two bands (5.4 and 2.3 kb), whereas hybridization with the HAX₄ probe detected three bands (11, 5.4, and 2.3 kb) (Fig. 7F). Since there is no *Bst*EII site in the 2.0-kb *Eco*RI fragment from λ 111 (data not shown), the 2.3-kb *Bst*EII fragment should be a junction fragment resulting from the presence of *Bst*EII sites in the 2.0-kb *Eco*RI *proto-amv* fragment and in the λ long arm. We can assume that the *Bst*EII site present in the 2.0-kb *Eco*RI fragment corresponds to the *Bst*EII site present in *amv* and that there is a *Bst*EII site in the λ long arm which is 1.3 kb upstream from its 3' terminus. The 5.4-kb *Bst*EII fragment encompasses the 2.0- and 2.1-kb *Eco*RI fragments, positioning a second *Bst*EII site 1.3 kb downstream from the 5' terminus of the 8.7-kb *Eco*RI *proto-amv* fragment. The 11-kb fragment detected with HAX₄ positioned in the λ short arm a *Bst*EII site 3.6 kb from the 5' end.

When a *Sma*I digest of λ 322112 DNA was hybridized to the SES3 probe, a 20-kb band was detected (Fig. 7E), whereas two bands (22 and 20 kb) hybridized with the HAX₄ probe (Fig. 7F). Thus, there is only one *Sma*I site within the chicken DNA insertion of λ 322112, presumably, as in *amv*, 150 base pairs downstream from the 5'-terminal *Eco*RI site of the 2.0-kb *Eco*RI *proto-amv* fragment.

Only one band (24 kb) was detected in the *Sal*I digest of λ 322112 DNA after hybridization with either the SES3 probe or the HAX₄ probe (Fig. 7E and F). The single band present after ethidium bromide staining (Fig. 7D) also confirmed the presence of a single *Sal*I site in this recombinant. Since λ DNA is not cleaved by *Sal*I (12), this site must be the one located 1.45 kb from the 5'-terminal *Eco*RI site of the 2.0-kb *Eco*RI *proto-amv* fragment (20).

(iii) ***Sma*I-*Eco*RI or *Sma*I-*Hind*III double digestion.** When a *Sma*I-*Eco*RI double digest of λ 322112 DNA was hybridized to the SES3 probe (Fig. 8B), a single 1.85-kb band was detected, confirming the position of the *Sma*I site 150 base pairs downstream from the *Eco*RI site in *proto-amv* (20), as in *amv* (23). Hybridization with HAX₄ detected the 1.85-kb band and the other two *Eco*RI *proto-amv* fragments (8.7 and 2.0 kb) (Fig. 8C).

Hybridization with SES3 of the *Sma*I-*Hind*III double digest of λ 322112 (Fig. 8B) revealed two fragments (20 and 1.6 kb). The 1.6-kb fragment confirmed the position of the *Hind*III and *Sma*I sites described above and in a previous study (20). The 20-kb fragment is a junction fragment with the λ long arm. Hybridization of the *Sma*I-*Hind*III double digest with HAX₄ (Fig. 8C)

FIG. 8. *Sma*I-*Eco*RI or *Sma*I-*Hind*III double digestion of λ 322112. DNA (0.5 μ g) from λ 322112 was digested either with *Sma*I and *Eco*RI (lanes a) or with *Sma*I and *Hind*III (lanes b). After ethidium bromide staining (A), the electrophoresed DNA fragments were transferred to nitrocellulose and hybridized with SES3 (B) or with HAX₄ (C). A mixture of *Hind*III-digested λ DNA and *Hae*III-digested ϕ X174 RF DNA was run as size markers.

revealed, in addition to the 1.6-kb band, the 1.3- and 5.2-kb *proto-amv* fragments generated by *Hind*III alone.

(iv) ***Sal*I-*Eco*RI or *Sal*I-*Hind*III double digestion.** When a *Sal*I-*Eco*RI double digest of λ 322112 DNA was hybridized to SES3 (Fig. 9B), two bands (1.45 and 0.95 kb) were detected, whereas three dark bands (1.45, 2.0, and 8.7 kb) and two fainter bands (0.95 and 0.6 kb) were detected with HAX₄ (Fig. 9C). The 1.45-kb band detected with SES3 confirmed that the *Sal*I site is located 1.45 kb downstream from the 5' end of the 2.0-kb *Eco*RI *proto-amv* fragment and that there is an intron between these two sites since the *Sal*I site is 350 base pairs downstream from the *Eco*RI site in *amv* (23). The faint 0.95-kb band detected with SES3 and HAX₄ indicates that a short *proto-amv* segment is present upstream from the *Eco*RI site which corresponds to the *amv* *Eco*RI site. The faint 0.6-kb band detected with HAX₄ represents another short *proto-amv* segment located downstream from the *Sal*I site in this small *Sal*I-*Eco*RI *proto-amv* fragment (20).

Hybridization with SES3 of the *Sal*I-*Hind*III double digest of λ 322112 (Fig. 9B) detected only

FIG. 9. *Sall*-*Eco*RI or *Sall*-*Hind*III double digestion of λ 322112. DNA (0.5 μ g) from λ 322112 was digested with *Sall* and *Eco*RI (lanes b) or with *Sall* and *Hind*III (lanes a). After staining with ethidium bromide (A), the fragments were transferred to nitrocellulose, and the blots were hybridized either with SES3 (B) or with HAX₄ (C). A mixture of *Hind*III-digested λ DNA and *Hae*III-digested ϕ X174 RF DNA was run as size markers.

the large band (19 kb), which was also detected with the same probe in the *Sall* digest. This confirmed that there is no *Hind*III site between the *Sall* site in *proto-amv* DNA and the 5'-terminal *Eco*RI site of the chicken DNA insertion in λ 322122. Hybridization with HAX₄ (Fig. 9C) revealed the two *Hind*III-specific 5.2- and 1.3-kb fragments in addition to the long 19-kb

fragment. The faint 0.4-kb band detected with HAX₄ corresponds to the short *amv* sequence present downstream from the *Sall* site in this *Sall*-*Hind*III *proto-amv* fragment (Fig. 7).

Restriction endonuclease analysis of λ *proto-amv* DNA recombinant clone 121. (i) *Sall*, *Eco*RI, *Hind*III, *Sma*I, or *Bam*HI digestion. The other newly isolated λ *proto-amv* recombinant clone, λ 121, was digested with either *Sall*, *Eco*RI, *Hind*III, *Sma*I, or *Bam*HI. After electrophoresis in 0.8% agarose gels and staining with ethidium bromide, the separated fragments were blotted onto nitrocellulose filter paper and hybridized with the ³²P-labeled SES3 probe. The blots were then thoroughly washed and rehybridized with the ³²P-labeled HAX₄ probe. Figure 10 shows that λ clone 121 contains the 5.4-kb *Eco*RI *proto-amv* fragment which is 5' proximal in the chicken *proto-amv* DNA domain, the 0.95-kb *Eco*RI fragment which has a short *proto-amv* sequence at its 3' end, and the 2.0-kb *Eco*RI fragment which contains two *proto-amv* segments. One of the latter two segments contains the *Sma*I site, and the other contains the *Sall* site, which correspond to the *Sma*I and *Sall* sites present in the viral insertion *amv*. Therefore, the chicken DNA insertion in λ 121 overlaps that of λ 1041 and that of λ 322112.

(ii) *Hind*III-*Eco*RI, *Hind*III-*Kpn*I, *Hind*III-*Bam*HI, *Hind*III-*Sma*I, *Eco*RI-*Sall*, or *Sma*I-*Kpn*I double digestion. The DNA of λ 121 was cleaved with different restriction enzyme combinations (*Hind*III and *Eco*RI, *Hind*III and *Kpn*I, *Hind*III and *Bam*HI, *Hind*III and *Sma*I, *Eco*RI and *Sall*, *Sma*I and *Kpn*I) and analyzed as described above. The results (Fig. 10) confirmed our previous restriction enzyme site mapping of this *proto-amv* region in C/O normal chicken DNA (20) and in the chicken DNA insertions of λ clones 1041 and 322112.

Location in chicken DNA of the 5'- and 3'-proximal *proto-amv* regions. To map more accu-

FIG. 10. Restriction endonuclease map of λ recombinant clone 121. DNA (0.5 μ g) from λ recombinant clone 121 was digested with either *Sall*, *Eco*RI, *Hind*III, *Sma*I, *Bam*HI, *Hind*III plus *Eco*RI, *Hind*III plus *Kpn*I, *Hind*III plus *Bam*HI, *Hind*III plus *Sma*I, *Eco*RI plus *Sall*, or *Sma*I plus *Kpn*I. After electrophoresis in 0.8% agarose gels, the gels were stained with ethidium bromide, photographed, blotted onto nitrocellulose, hybridized to ³²P-labeled SES3 probe, autoradiographed, washed thoroughly, rehybridized to ³²P-labeled HAX₄ probe, and autoradiographed again. The cross-hatched bars represent the λ arms generated by *Eco*RI digestion, and the solid boxes represent *proto-amv* segments. The arrows represent recognition sites for the following restriction endonucleases: *Bam*HI (B), *Kpn*I (K), *Eco*RI (E), *Sma*I (Sm), *Hind*III (H), and *Sall* (Sa).

ately the 5'-proximal *proto-amv* segment carried by λ 1041 and the 3'-proximal segment carried by λ 533, the corresponding 5.4- and 8.7-kb *EcoRI* fragments were subcloned and analyzed further. The fragments were purified by electrophoresis in 0.8% agarose gels and electroelution (19) and then recloned in pBR322 after insertion at the *EcoRI* site (see above). In the resulting clones, P542 and P873 (Fig. 1 and 11),

the orientation of the chicken DNA insertions with respect to pBR322 was determined by single and double digestions with endonucleases *Bam*HI, *Hind*III, and *Eco*RI (data not shown). In clone P542 the 5.4-kb fragment was oriented with its 3' terminus toward the *Bam*HI site of pBR322, whereas in clone P873 the 8.7-kb fragment was inserted in the opposite orientation.

(i) Restriction analysis of plasmid *proto-amv*

FIG. 11. Restriction endonuclease analysis of pBR322 *proto-amv* recombinant clones p542 and P873. (A) DNA (0.5 μ g) from clone P542 was digested with either *Pvu*II (lane a) *Hae*II (lane b), *Acc*I (lane c), or *Pst*I (lane d). After electrophoresis in 0.8% agarose gels, the blots were hybridized to HAX₄. The restriction site map of P542 shows pBR322 in linear form (cross-hatched bars) after cleavage at the single *Pst*I site (position 3,612). The position of the two *Hae*II sites encompassing the *proto-amv* segment (solid box) is approximate. (B) DNA (0.5 μ g) from clone P873 was digested with either *Ball*I (lane a), *Xho*I (lane b), or *Xba*I (lane c). After electrophoresis in 0.8% agarose gels, the blots were hybridized to HAX₄. A mixture of *Hind*III-digested λ DNA and *Hae*III-digested ϕ X174 RF DNA was run as size markers. The restriction map of P873 shows pBR322 in linear form (cross-hatched bars) after cleavage at the single *Ball*I site (position 1,445). The position of the *Xho*I site in the chicken DNA insertion could not be determined accurately.

DNA recombinant clone P542. When P542 DNA digested with *Pst*I was hybridized with HAX₄, a 4.4-kb fragment was detected (Fig. 11A). The location (clockwise) of the single *Pst*I site 3.6 kb from the *Eco*RI site in pBR322 (32) and the sizes of the *Pst*I fragments detected after ethidium bromide staining (data not shown) localized within a 0.8-kb *Pst*I-*Eco*RI *proto-amv* fragment the *amv* sequences between the *Hae*II and *Eco*RI sites.

There are two *Acc*I sites in pBR322, one at position 651 and the other at position 2,246 (32). Hybridization of an *Acc*I digest of P542 DNA with HAX₄ detected two bands (3.9 and 2.3 kb) (Fig. 11A). The 2.3-kb fragment positions an *Acc*I site 1.65 kb upstream from the 3'-terminal *Eco*RI site of the chicken insertion in P542. The 3.9-kb fragment presumably reflects incomplete digestion of the *Acc*I sites (i.e., $2.3 + 1.6 = 3.9$ kb).

Hybridization of a *Hae*II digest of P542 DNA with HAX₄ revealed a 0.87-kb fragment (Fig. 11A). Thus, there is at least one *Hae*II site on each side of the *proto-amv* segment, but the position of these *Hae*II sites cannot be determined with the data currently available.

A *Pvu*II digest of P542 DNA revealed a 3.4-kb fragment after hybridization with HAX₄ (Fig. 11A). The single *Pvu*II site in pBR322 at 2.1 kb (clockwise) from the *Eco*RI site positions a *Pvu*II site 1.3-kb upstream from the 3'-terminal *Eco*RI site of the chicken DNA insertion.

(ii) **Restriction analysis of plasmid *proto-amv* DNA recombinant clone P873.** Hybridization of the *Xba*I or *Xho*I digest of P873 DNA with HAX₄ detected a single 13-kb fragment (Fig. 11B). This indicates that a single site for each of these two endonucleases is present within the chicken DNA insertion since these two enzymes do not cleave pBR322. The position of the *Xba*I site 1.7 kb from the 5'-terminal *Eco*RI site within the 8.7-kb *Eco*RI *proto-amv* fragment was determined previously from *Hind*III-*Xba*I and *Bam*HI-*Xba*I double digests of the λ *proto-amv* recombinant clones which carry this *proto-amv* fragment.

In *amv* there is a *Bal*I site 80 base pairs upstream from the *Xba*I site (23). Two fragments (3.3 and 2.0 kb) were detected with HAX₄ in a *Bal*I digest of clone P873 DNA (Fig. 11B). Thus, the *Bal*I site is located within the *proto-amv* sequence. The 3.3-kb fragment locates a *Bal*I site in the chicken DNA insertion 1.9 kb downstream from the 5'-terminal *Eco*RI site since there is a *Bal*I site at position 1,445 in pBR322 (32). The 2.0-kb *Bal*I *proto-amv* fragment locates a second *Bal*I site 2.0 kb downstream from the first one. The large (>10-kb) band appears to be due to incomplete enzymatic digestion.

The arrangement of the *proto-amv* sequences

and their major restriction enzyme sites are shown in Fig. 12, along with the chicken DNA insertions of the various λ recombinant clones analyzed in this study.

DISCUSSION

In the chicken genome the *proto-amv* domain covers 8 to 9 kb of DNA. The cellular genetic elements corresponding to the information present in *amv* are contained within six major *proto-amv* segments which are interrupted by at least five DNA regions lacking homology with *amv* (Fig. 12).

The 5'-terminal *proto-amv* segment carried in λ clones 1041 and 121 is located in a 0.8-kb DNA fragment bounded by a *Pst*I site and an *Eco*RI site. As previously discussed (20), it appears unlikely that this *Eco*RI site corresponds to the *amv* internal *Eco*RI site (19, 23). Also, the lack of a specific probe for the *amv* region located between the 5' terminus and the *Hae*II site did not permit us to determine whether the *proto-amv* region located upstream from the *Hae*II site is contiguous with the rest of this *proto-amv* segment. However, we do know from hybridization with the AMV *Kpn*I-*Xba*I fragment (19) that all of the 5'-terminal sequences of *proto-amv* are present within the 5'-terminal 2.2-kb *Hind*III-*Eco*RI fragment.

The existence and location next to the first *proto-amv* segment of a nonhomologous region containing the 0.95-kb *Eco*RI intervening fragment detected in C/O normal DNA (20) have been confirmed by the presence of this region in λ *proto-amv* clones 121 and 322112. A short stretch of *proto-amv* sequences is present within this 0.95-kb *Eco*RI fragment, as demonstrated by its faint hybridization to the HAX₄ probe (Fig. 9C).

The next two *proto-amv* segments (segments 2 and 3) located within the 2.0-kb *Eco*RI fragment are carried by λ clones 121 and 322112. Segment 2 contains the *Eco*RI, *Bst*EII, and *Sma*I sites which are present in *amv* (19, 23). Segment 3 contains the *Sal*I site which is present in *amv* 350 base pairs downstream from the *Eco*RI site. The results obtained with normal C/O DNA (20) and λ clone 322112 (Fig. 9C) show that there is a small stretch of *proto-amv* sequences downstream from the *Sal*I site in segment 3.

proto-amv segments 4 and 5 carried by λ clones 111, 233, and 322112 are located in the 2.1-kb *Eco*RI fragment adjacent to the 2.0-kb *Eco*RI fragment. There is a *Hind*III site between segments 3 and 4 and another *Hind*III site between segments 4 and 5. Segment 5, which was not detected when total chicken DNA was analyzed (20), appears to be relatively short because of its low level of hybridization with the HAX

FIG. 12. Organization of *proto-amv* sequences in leukemic DNA. (A) The *amv* insertion of the AMV provirus is subdivided into regions corresponding to the various hybridization probes used in this study. The restriction map of the leukemic chicken DNA region containing *proto-amv* sequences shows the arrangement of the different *proto-amv* segments identified in this paper. The question mark at the 5' end of *amv* indicates that we do not know whether this 5' segment is contiguous with the *Hae*III site in chicken DNA. The location of other *Bal*I sites besides the two shown defining the 3' boundary of the *proto-amv* domain has not been determined. The length scales for the *amv* insertion and chicken DNA are different. (B) The chicken DNA insertions of the different λ *proto-amv* recombinant clones analyzed in this study are presented along with their *proto-amv* contents. The arrows indicate *Eco*RI sites.

probe (Fig. 5). The amplification of *proto-amv* sequences provided by cloning in the λ vector made the detection of this segment possible.

The sixth 3'-terminal *proto-amv* segment (segment 6) identified in this study is present in λ clones 111, 233, 533, 1132, and 322112. There is an *Xba*I site within the upstream intervening region very close to this segment, which contains a *Bal*I site near its 3' terminus. The position of the *Bal*I site locates more precisely *proto-amv* segment 6 within the 3'-terminal 4.4-kb *Eco*RI-*Hind*III fragment and places an upper limit of approximately 9 kb on the size of the *proto-amv* domain. A *Bal*I site is located within *amv* near the 3' terminus 80 base pairs upstream from the viral *Xba*I site, which is 30 base pairs downstream from the 3' recombination point of the insertion in the AMV genome (23). There is an *Xba*I site at the same location in the free linear DNA intermediate and in the cloned proviral DNA of helper myeloblastosis-associated virus type 1 (2; unpublished data). The *Xba*I site located just in front of *proto-amv* segment 6 upstream from the *Bal*I site or the *Xba*I site located approximately 6.5 kb downstream from the *Bal*I site in a 2.7-kb *Eco*RI fragment which does not hybridize with HAX₄ cannot correspond to the *Xba*I site 30 base pairs downstream

from *amv* in the AMV genome. Therefore, in the AMV genome the 3'-proximal *Xba*I site which appears to contain the termination codon for the putative AMV oncogene is part of helper virus sequences. Also, 34 of the last 35 bases, including the termination codon at the 3' end of the AMV transforming gene, are the same as the bases present at the 3' end of the envelope gene of Rous sarcoma virus (23). Nucleotide sequence analysis of the recently isolated myeloblastosis-associated virus type 1 provirus should determine unequivocally the number of helper virus nucleotides which are part of the AMV leukemogenic information.

The nucleotide sequence of *amv* (23) reveals the following: (i) the 5'-terminal region is 84 base pairs long, terminating with TAG and apparently representing the 3' terminus of a protein-encoding sequence; (ii) this is followed by 340 base pairs (up to the *Eco*RI site) which do not contain an initiation codon or a termination codon but contain transcription regulatory elements; and (iii) after the *Eco*RI site starting with ATG there is an open reading frame of 762 base pairs up to the 3' recombination point with helper virus sequences which provide an additional 33 base pairs before reaching a termination codon. Thus, the *amv* insertion in AMV may represent the 3'-

terminal portion of one cellular gene followed by an intron and then by the 5' portion of a second gene. Correspondingly, the first *proto-amv* segment (Fig. 12), which represents *amv* sequences upstream from the *EcoRI* site, should contain the 3'-terminal portion of one gene and the adjacent intron. The other five *proto-amv* segments could represent part of the exons of a second gene. If that were the case, the *amv* insertion would not represent a complete cellular gene or a complete viral oncogene.

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