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

I. Restriction Enzyme Analysis of Total DNA from Normal and Leukemic Cells

BERNARD PERBAL AND MARCEL A. BALUDA*

UCLA School of Medicine and Jonsson Comprehensive Cancer Center, Los Angeles, California 90024

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Hybridization probes consisting of cloned DNA recombinants which represent different regions of the leukemogenic sequence (*amv*) from avian myeloblastosis virus were used to carry out a more detailed restriction endonuclease analysis of the homologous sequences (*proto-amv*) present in normal and leukemic chicken DNA. The results show that four large introns interrupt the normal cellular *proto-amv* sequences and that there is no major rearrangement of these sequences in leukemic myeloblasts.

The leukemogenic potential of avian myeloblastosis virus (AMV) has been associated with a sequence (*amv*) of approximately 1.2 kilobase pairs (kb) which has replaced the viral *env* gene (18, 19). The *amv* insert is homologous to a unique chicken DNA region (*proto-amv*) which is interrupted by unrelated DNA sequences (12). Introns are present within the cellular homologs of most viral oncogenes. For instance, the chicken cellular homolog of the *src* gene of Rous sarcoma virus (Prague C strain) contains at least five introns (16), and cat DNA sequences homologous to the oncogene of the Snyder-Theilen feline sarcoma virus contain at least three introns (9).

To further characterize the arrangement of the *proto-amv* sequences in normal chicken DNA, DNA recombinant clones representing different regions of *amv* were prepared and used as hybridization probes with C/O chicken DNA treated with various restriction endonucleases either singly or in combinations. A similar analysis was performed with DNA from leukemic chicken myeloblasts. These studies revealed that in normal chicken DNA, the *proto-amv* sequences are interrupted by at least four large introns, and that there is no major reorganization of these sequences in leukemic DNA.

MATERIALS AND METHODS

Purification of total DNA. Purification of pBR322 DNA was performed as described by Curtiss et al. (8). High-molecular-weight DNA (4.5 kb or larger) was purified from C/O and C/E whole chicken embryos or tissues as described earlier (1).

Purification of DNA fragments. The DNA fragments obtained by restriction enzyme treatment of pBR322 hybrid clones were purified by electroelution as already described (12).

Preparation of Southern blots. The DNA fragments obtained after electrophoresis in agarose gels were blotted onto nitrocellulose Millipore filters (Millipore Corp., Bedford, Mass.) under the conditions described earlier (12, 17).

Nick translation. Conditions used for ^{32}P labeling of DNA fragments by nick translation (13) were reported earlier (12).

Cloning. *Escherichia coli* HB101 (4) was the recipient and pBR322 was the vector in transformation experiments. Ligation conditions and transformation procedures were previously described (12). Screening of the transformant clones for the nature of their plasmid DNA content was performed by a modification of the method described by Birnboim and Doly (3).

Individual transformants were grown overnight at 37°C in 5 ml of TYE medium (10 g of Tryptone [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract, and 5 g of NaCl per liter) containing 100 μg of ampicillin per ml. A 1.5-ml volume of the cell suspensions was then transferred to an Eppendorf tube and spun for 30 s in an Eppendorf centrifuge. After aspiration of the supernatant, the cell pellet was blended in a Vortex mixer for 15 s and suspended in 100 μl of a freshly prepared lysozyme solution (25 mM Tris-hydrochloride [pH 8.0]-10 mM EDTA-50 mM glucose-5 mg of lysozyme per ml [Sigma Chemical Co., St Louis, Mo.]). After 15 min of incubation at 4°C, 200 μl of 0.2 N NaOH-1% sodium dodecyl sulfate was added, and the solution was mixed vigorously. After an additional 5 min on ice, 150 μl of 3 M sodium acetate (pH 5.0) was added, and the mixture was left at 4°C for 1 h. The precipitate which formed after addition of the high salt solution was spun down for 10 min at 4°C, and the supernatant was extracted once with 400 μl of a 1:1 phenol-chloroform-isoamyl mixture (chloroform-isoamyl is 24:1) and twice with 400 μl of chloroform-isoamyl

(24:1). Nucleic acids were precipitated at −70°C for 30 min after the addition of 2 volumes of absolute ethanol, pelleted by centrifugation, and suspended in 60 μl of sterile distilled water. Usually, 5 to 10 μl of this suspension was used to run analytical digests in the presence of 10 μg of RNase A.

Colony hybridization. The method described by Grunstein and Hogness (11) was used. The washing and sterilization of the filters before seeding with bacteria could be omitted without altering the quality of the hybridization obtained if ampicillin plates were used.

Hybridization. The DNA blots and the colony-bearing filters were hybridized as described previously (2, 12).

Preparation of *amv*-specific subclones. The pBR HAX_4 clone (12) was used to prepare a subclone containing the *amv* sequences located between the *Hae*II and the *Eco*RI sites found within the 1-kb *amv* insert in HAX_4. The 1.0-kb *amv* fragment was isolated from pBR HAX_4 by *Bam*HI digestion, electrophoresis in a 0.8% agarose gel, and electroelution. It was then digested with *Eco*RI endonuclease, and the two resulting fragments (0.80 and 0.20 kb) were separated on a 2% agarose gel. The 200-base pair fragment corresponding to the 5′ portion was then cloned in pBR322 EB previously electroeluted from a 0.8% agarose gel after double digestion with *Bam*HI and *Eco*RI endonucleases. Screening of the resulting tetracycline-sensitive colonies revealed that some transformants carried the expected size insert, and hybridization with ^{32}P-labeled HAX_4 showed that they contained the specific *amv* fragment. One of these transformants (pBR EB3) was used in this work.

Clones SES3 and SX12, which contain the *amv* sequences located between the *Eco*RI-*Sal*I sites and the *Sal*I-*Xba*I sites, respectively, were prepared from the pBR-EX11 clone (12).

Digestion of pBR-EX11 with *Sal*I generated two DNA fragments (0.75 and 4.4 kb) which were separated by electrophoresis in a 1% agarose gel and recovered by electroelution. The 4.4-kb fragment which contained the 3′ portion between *Sal*I and *Xba*I also contained the pBR322 sequences located between the *Bam*HI and *Sal*I sites (including the pBR322 replicon). Therefore, the electroeluted DNA fragment was self-ligated and used directly to transform *E. coli* HB101. The transformants were shown to contain *amv*-specific sequences if screened by colony hybridization with purified ^{32}P-labeled HAX_4 DNA. One of these clones (SX12) was used in subsequent experiments.

The other fragment (0.75 kb) generated by *Sal*I treatment of pBR-EX11 was subcloned in pBR322 previously digested with *Sal*I and then was treated with alkaline phosphatase under conditions described earlier (12). Tetracycline-sensitive clones were analyzed by both miniscreening and colony hybridization with ^{32}P-labeled HAX_4 DNA. One of the clones having the correct insert (pBR SES3) was used as a specific probe for the *amv* sequences located between the *Eco*RI and *Sal*I sites.

The derivation of these new *amv* subclones is summarized in Fig. 1.

Preparation of the *proto-amv* E3 probe. Probe E3 was derived from the λ-chicken recombinant clone no. 111 which contains two *Eco*RI fragments (2.0 and 8.7 kb) of *proto-amv* sequences corresponding to the 3′ end of the *amv* sequence (12). The 8.7-kb *Eco*RI fragment was purified by electroelution after digestion of λ111 DNA with *Eco*RI endonuclease and electrophoresis in a 0.8% agarose gel. The 8.7-kb electroeluted fragment (E3) was ^{32}P labeled by nick translation to obtain hybridization probe.

Physical and biological containment. This work was carried out at the P2-EK2 containment levels according to the revised guidelines of the National Institutes of Health.

RESULTS

Analysis of normal C/O chicken DNA. (i) Hybridization with HAX_4 probe. To determine the relative position in normal chicken DNA of the *Eco*RI, *Hin*dIII, and *Bam*HI endonuclease sites which generate *proto-amv* DNA fragments, double enzymatic digestions were performed, and the resulting Southern blots were hybridized to the ^{32}P-labeled HAX_4 probe which contained most of *amv* but no viral sequence. Single *Eco*RI and *Hin*dIII DNA digests were included within the same gel as internal controls in addition to standard size markers.

The blot from a *Hin*dIII + *Eco*RI digest

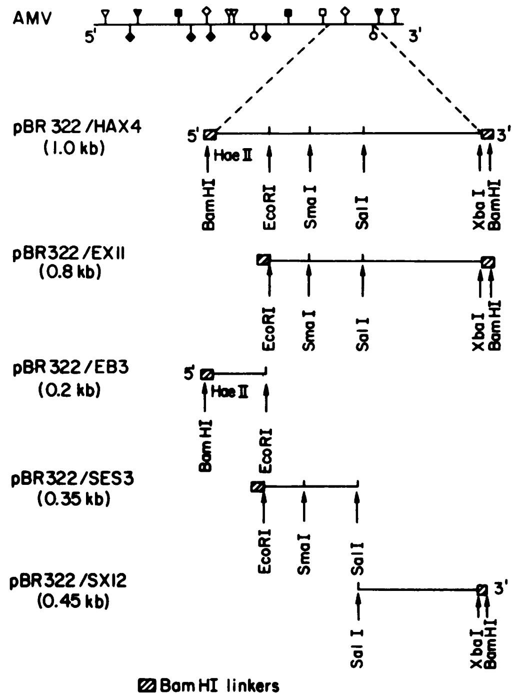

FIG. 1. Derivation of the *amv*-specific subclones. The restriction endonuclease sites in AMV are mapped as previously reported (12, 18). Symbols: ▽, *Hin*dIII; ◆, *Bam*HI; ▼, *Xho*I; ■, *Bgl*II; ◇, *Eco*RI; ○, *Xba*I; □, *Kpn*I. The subcloning of HAX_4 and EX11 from AMV has been described (12). In probes HAX_4 and EB3, the *Hae*II site originally present in *amv* has been removed during *Bam*HI linker ligation.

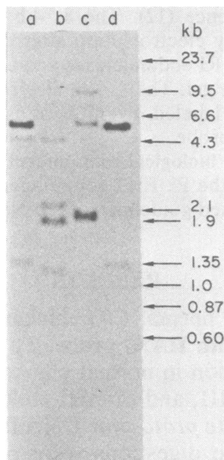

FIG. 2. Hybridization of restriction enzyme-treated C/O chicken DNA with HAX_4. C/O chicken DNA was digested with either *Hin*dIII (d), *Eco*RI (c), successively with *Hin*dIII and *Eco*RI (b), or with *Hin*dIII and *Bam*HI (a). The digested DNA was electrophoresed in 0.8% agarose, transferred to nitrocellulose filters (Southern blots), and hybridized to ^{32}P-labeled HAX_4 DNA. A mixture of *Hin*dIII-digested λ DNA and *Hae*III-digested φX174 RF DNA was run in parallel and used as molecular size markers, expressed in kilobase pairs (kb).

showed four bands of 1.2, 1.9, 2.2, and 4.3 kb (Fig. 2, lane b), whereas *Eco*RI or *Hin*dIII alone yielded the band pattern previously reported (12). Three fragments (2.1, 5.4, and 8.7 kb) were detected in the *Eco*RI digest (Fig. 2, lane c), and two fragments (1.3 and 5.2 kb) were detected in the *Hin*dIII digest (Fig. 2, lane d). The 1.3-kb *Hin*dIII fragment had previously been estimated to be 1.4 kb (12).

The *Bam*HI + *Hin*dIII digest revealed three fragments (1.3, 4.3, and 5.2 kb) with the ^{32}P-labeled HAX_4 probe (Fig. 2a). Previously (12), we had reported that *Bam*HI digestion of C/O DNA generates two *proto-amv* fragments (5.4 and 19.6 kb).

A similar hybridization pattern was obtained with DNA purified from embryonic fibroblasts, spleen cells, or yolk sac cells obtained from C/E chickens (data not shown).

The proportion of *proto-amv* sequences present within the *Eco*RI and *Hin*dIII fragments cannot be accurately determined, and consequently the relative intensity of the bands cannot be used for quantitative estimations. Nevertheless, the strong intensity of the 2.1-kb *Eco*RI band suggested the possibility that it represents hybridization of HAX_4 with two *proto-amv* DNA fragments of similar size. Previously, we had shown that the *Eco*RI 2.1-kb fragment detected with HAX_4 included sequences located in *amv* beyond the *Sal*I site towards the 3′ end. Therefore, an *Eco*RI + *Sal*I digest was performed on total C/O DNA to determine whether this 2.1-kb band corresponded to a doublet. The results showed that in addition to the *Eco*RI 8.7- and 5.4-kb bands, two other bands, of 1.45 and 2.0 kb, were present (Fig. 3). The low intensity of the 1.45-kb band relative to that of the 2.0-kb band presumably resulted from inefficient cleavage of the 2.0-kb fragment with *Sal*I. In three independent *Sal*I treatments, it was not possible to obtain complete digestion if total chicken DNA was used as substrate. The 1.45-kb band also hybridized with the SES3 probe, which consisted of the *amv* sequences located between the *Eco*RI and *Sal*I sites (data not shown). This positioned the *Sal*I site at 1.45 kb downstream from the *Eco*RI site within the corresponding *proto-amv* region, thereby revealing an intron between these two enzyme sites. The position of the *Sal*I site was confirmed by analysis of λ *proto-amv* recombinant clones presented in our second paper on this subject (B. Perbal, J. M. Cline, R. L. Hillyard, and M. A. Baluda, submitted for publication). The presence of the intact 2.0-kb fragment and the 1.45-kb fragment is in agreement with the possibility that the 2.1-kb band generated by *Eco*RI alone is a doublet.

To determine the location of the additional *Eco*RI 2.0-kb *proto-amv* fragment, in normal chicken DNA, several double enzymatic digestions were performed on C/O DNA, and the resulting blots were hybridized with DNA probes representing different portions of the *amv* sequence. The preparation and location of

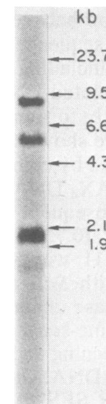

FIG. 3. Double digestion of C/O DNA with *Sal*I + *Eco*RI. C/O DNA was digested with *Sal*I and *Eco*RI endonucleases in the *Eco*RI running buffer. After electrophoresis in a 0.8% agarose gel and transfer to nitrocellulose, the resulting blot was hybridized to ^{32}P-labeled HAX_4 DNA. *Hin*dIII-digested λ DNA was run as size markers.

TABLE 1. Hybridization of restriction enzyme fragments from C/O chicken DNA with different *amv* probes

Enzymes	Fragments (kb) detected with *amv* probe:			
	HAX_4	EB3	SES3	SX12
*Hin*dIII + *Eco*RI	1.2, 1.9, 2.2, 4.3	2.2	1.9	1.2, 1.9, 4.3
*Bam*HI + *Eco*RI	2.0, 5.4	5.4	2.0	2.0, 5.4
*Sma*I + *Eco*RI	2.0, 4.9, 8.7	4.9	2.0	2.0, 8.7
*Hin*dIII + *Bam*HI	1.3, 4.3, 5.2	5.2	5.2	1.3, 4.3

these probes within *amv* are described in Materials and Methods and shown in Fig. 1.

(ii) Hybridization of double enzymatic digests with subclones of HAX_4. Four different double digests were set up. In addition to *Hin*dIII, *Eco*RI, and *Bam*HI endonucleases, *Sma*I was used because of the location of its recognition site 150 base pairs towards the 3′ terminus from the *Eco*RI site in *amv* (12, 14), The double digests were electrophoresed in 0.8% agarose gels, transferred to nitrocellulose, and hybridized with one of the probes HAX_4, EB3, SES3, or SX12 (for a summary, see Table 1).

(i) Among the four *proto-amv* fragments generated in the *Hin*dIII + *Eco*RI digest (Fig. 4, lanes d), the 2.2-kb fragment hybridized with the *amv* sequences located between the *Hae*II and *Eco*RI sites (probe EB3), whereas the 1.9-kb fragment hybridized with both the SES3 and SX12 probes which include *amv* sequences located between the *Eco*RI and *Xba*I sites. The faintness of the 1.9-kb band detected with the SX12 probe indicates that only a short stretch of *amv* sequences which lay on the 3′ side of the *Sal*I site of *amv* was present in this fragment. The two other fragments (1.2 and 4.3 kb) generated in the *Eco*RI + *Hin*dIII digest hybridized only with the SX12 probe. These findings indicated that the 3′ portion of *amv* found between the *Sal*I and *Xba*I sites was distributed in three separate *proto-amv* fragments generated by *Eco*RI and *Hin*dIII double digestion, i.e., 1.9, 1.2, and 4.3 kb. Therefore, this portion of *amv* must be located in at least two domains of exon in *proto-amv*.

A comparison of the sizes of the DNA fragments detected after a single digestion with either *Eco*RI or *Hin*dIII (12) with the results obtained above allows us to order the fragments obtained in the *Hin*dIII + *Eco*RI digest as follows from the 5′ to the 3′ end: 2.2, 1.9, 1.2, and 4.3 kb. The relative order of the 1.2- and 4.3-kb fragments was deduced from the order of the *Hin*dIII fragments: 5.4, 1.3, and 5.4 kb, assuming that the 1.2-kb fragment in the double *Eco*RI + *Hin*dIII digest corresponds to the *Hin*dIII 1.3-kb fragment. This was confirmed by the results obtained with the *Hin*dIII + *Bam*HI double digestion (see below). The appearance of the 2.2-kb fragment fixed the position of a *Hin*dIII

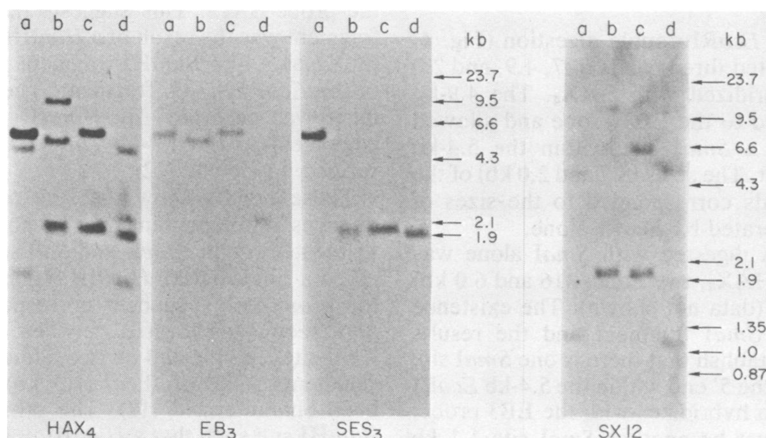

FIG. 4. Hybridization of C/O DNA double digests with different amv probes. C/O DNA was digested successively by either *Hin*dIII and *Eco*RI (d), *Bam*HI and *Eco*RI (c), *Sma*I and *Eco*RI (b), or *Hin*dIII and *Bam*HI (a). After electrophoresis in 0.8% agarose gels and transfer to nitrocellulose, the blots were hybridized to the indicated probes ^{32}P labeled by nick translation. The size markers were either *Hin*dIII-digested λ DNA or a mixture of *Hin*dIII-digested λ DNA and *Hae*III-digested φX174 RF DNA. The gel used for SX12 hybridization was electrophoresed for a longer period than were the other gels.

site within the 5′-proximal 5.4-kb *Eco*RI fragment, and the presence of the 4.3-kb fragment fixed the position of a *Hind*III site within the 3′-proximal 8.7-kb *Eco*RI fragment.

Since only two bands (5.2 and 1.3 kb) were detected if a *Hind*III digest of C/O DNA was hybridized to HAX_4 (see above), the present results suggest that there is a second 3′-proximal *Hind*III fragment of 5.2 kb which overlaps with the *Eco*RI 8.7-kb fragment and includes the *Hind*III-*Eco*RI 4.3-kb fragment. This would place most of the 1.3-kb *Hind*III fragment within one of the two *Eco*RI fragments of 2.0 and 2.1 kb detected with HAX_4 and the 1.9-kb *Eco*RI-*Hind*III fragment within the other one.

(ii) When a double *Bam*HI + *Eco*RI digest (Fig. 4, lanes c) of C/O DNA was hybridized to HAX_4 ^{32}P-labeled DNA, two very dark bands (5.4 and 2.0 kb) were detected. The 5.4-kb band was probably a doublet consisting of the 5′-proximal 5.4-kb fragment detected after digestion with *Eco*RI alone and the 3′-proximal 5.4-kb fragment detected after digestion with *Bam*HI alone (12). The 2.0-kb band was also a doublet consisting of the 2.0-kb fragment generated by *Eco*RI alone and a 2.0-kb fragment resulting from the presence of a *Bam*HI site near the 3′ end of the 2.1-kb fragment also generated by *Eco*RI alone.

Hybridization of the *Bam*HI + *Eco*RI double digest with the probe EB3 revealed the 5′-proximal 5.4-kb *Eco*RI fragment, whereas hybridization with the probe SES3 revealed only a 2.0-kb fragment. The probe SX12 hybridized as expected with two fragments of 2.0 and 5.4 kb, the latter presumably corresponding to the 3′-proximal *Bam*HI fragment previously described (12).

(iii) *Sma*I + *Eco*RI double digestion (Fig. 4, lanes b) generated three bands (8.7, 4.9, and 2.0 kb) which hybridized with HAX_4. The 4.9-kb band hybridized to the EB3 probe and allowed us to localize a *Sma*I site within the 5.4-kb *Eco*RI fragment. The sizes (8.7 and 2.0 kb) of the two other bands corresponded to the sizes of fragments generated by *Eco*RI alone.

If C/O DNA digested with *Sma*I alone was hybridized to HAX_4, two bands (16 and 6.0 kb) were detected (data not shown). The existence of the 6.0-kb *Sma*I fragment and the results given above establish that there is one *Sma*I site at 0.5 kb from the 5′ end within the 5.4-kb *Eco*RI fragment which hybridizes with the EB3 probe. Also, there must be another *Sma*I site 1.1 kb outside the 3′ end of the same *Eco*RI fragment since a 4.9-kb fragment which hybridizes with the EB3 probe was generated by a double *Sma*I + *Eco*RI digestion. Because the 2.0-kb *Sma*I-*Eco*RI fragment hybridized with the SES3 probe, it contains *amv* sequences located between the *Eco*RI and *Sal*I sites and must correspond to the 1.9-kb *Eco*RI-*Hind*III fragment which also hybridizes to the SES3 probe. We can assume that as in *amv*, the *Sma*I site in this *proto-amv* region is located 150 base pairs to the 3′ side of the *Eco*RI site. Therefore, this *Eco*RI site would be located at 0.95 kb on the 3′ side of the *Eco*RI site which is at the 3′ end of the 5′-proximal 5.4-kb *Eco*RI fragment. This was determined as follows: *Sma*I-*Sma*I (6.0 kb) minus *Sma*I-*Eco*RI (4.9 kb) minus *Eco*RI-*Sma*I (0.15 kb) equals *Eco*RI-*Eco*RI (0.95 kb). Thus, there is an extra *Eco*RI site within the *proto-amv* region under discussion, establishing the existence of another intron consisting of at least 0.95 kb between two *Eco*RI sites. A similar conclusion can be drawn from the results obtained with the *Hind*III + *Bam*HI double digestion of C/O DNA. The existence of the 0.95-kb *Eco*RI fragment was confirmed by analysis of λ *proto-amv* recombinant clones (Perbal et al. submitted for publication).

(iv) Double digestion with *Hind*III + *Bam*HI (Fig. 4, lanes a) and hybridization to HAX_4 gave rise to three bands of 5.2, 4.3, and 1.3 kb. The 5.2-kb band, which also appeared after digestion with *Hind*III alone, was detected after hybridization with the EB3 and SES3 probes, confirming the presence of this *Hind*III fragment on the 5′ side of the *proto-amv* sequences. This 5.2-kb *Hind*III fragment was previously shown to carry *proto-amv* sequences common to both the 5.4- and the 2.0-kb *Eco*RI fragments (12). This observation also confirms that there is no *Bam*HI site in the 5.2-kb *Hind*III fragment (12).

The 4.3- and 1.3-kb fragments generated by *Hind*III + *Bam*HI digestion were detected by the probe SX12. This suggests that the 4.3-kb fragment was the result of a *Hind*III cut in the 3′-proximal 5.4-kb *Bam*HI fragment which is part of the *Eco*RI 8.7-kb fragment. The 1.3-kb fragment was generated by *Hind*III alone and is equivalent to the 1.4-kb *Hind*III fragment described previously (12).

These results show that the *proto-amv* sequences corresponding to the portion of *amv* delineated by the *Hae*II and *Sal*I sites are located on a single 5.2-kb *Hind*III fragment, whereas the *proto-amv* sequences corresponding to the *amv* sequences located between the *Sal*I and *Xba*I sites are located on two additional *Hind*III fragments. One of them (1.3 kb) has already been characterized (12). The other contains a *Bam*HI site such that a *Hind*III + *Bam*HI digestion generates a 4.3-kb fragment which hybridizes with the SX12 probe. Since only two *Hind*III bands (5.2 and 1.3 kb) were detected by hybridization with HAX_4, the 5.2-kb band must be a doublet. This was confirmed by hybridization of *Hind*III-digested C/O DNA with the

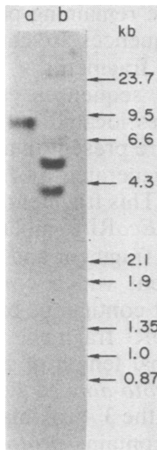

FIG. 5. Hybridization of *Eco*RI- or *Hin*dIII-digested C/O DNA with the *proto-amv* E3 probe. C/O DNA digested with either *Eco*RI (a) or *Hin*dIII (b) was electrophoresed in agarose gel, Southern blotted, and hybridized to ^{32}P-labeled E3 DNA, which represents the 3' end of the *proto-amv* sequences. A mixture of *Hin*dIII-digested λ DNA and *Hae*III-digested φX174 RF DNA was used as molecular size markers.

proto-amv E3 probe which corresponds to the 3'-terminal *amv* sequences (see above).

(iii) Hybridization of C/O DNA with *proto-amv* E3 probe. C/O DNA was digested with *Hin*dIII or *Eco*RI endonuclease, electrophoresed in a 0.8% agarose gel, blot transferred, and hybridized to ^{32}P-labeled E3 DNA. As expected, the 8.7-kb *Eco*RI fragment was detectable (Fig. 5a). However, the E3 probe hybridized to two DNA fragments (5.2 and 4.2 kb) in C/O DNA digested with *Hin*dIII (Fig. 5b). This confirms that there are two 5.2-kb *Hin*dIII fragments, one of which contains the *proto-amv* sequences present in the 8.7-kb *Eco*RI fragment. The 4.2-kb band must represent hybridization with non-*proto-amv* cellular DNA sequences present within the *Eco*RI 8.7-kb fragment. These data allow us to position the *Eco*RI, *Hin*dIII, *Sma*I, *Bam*HI, and *Sal*I sites within the region of normal C/O DNA which contains the *proto-amv* sequences (see Fig. 7).

Analysis of leukemic DNA. To determine whether there was a similar arrangement of *proto-amv* sequences in leukemic cells, DNA purified from peripheral blood myeloblasts of leukemic C/E chicken no. 21710 was treated with various restriction endonucleases and analyzed with *amv* probes. This DNA was the source of the λ recombinant library from which the AMV provirus recombinant clone λ11A1-1 was isolated (18). Knowledge of the location of many endonuclease sites within the AMV provirus (12, 14, 18) permits us to identify the AMV fragments which should hybridize to the HAX_4 probe.

Hybridization of *Hin*dIII-digested leukemic DNA (Fig. 6a) to the HAX_4 probe gave rise to three bands (5.2, 4.3, and 1.3 kb). The 4.3-kb fragment was not generated from normal DNA and corresponded to the 3'-proximal *Hin*dIII fragment of the AMV genome.

After *Eco*RI digestion of leukemic DNA, two AMV fragments hybridized with the HAX_4 probe (4.4 and 3.6 kb) in addition to the three *proto-amv* fragments (8.7, 5.4, and 2.0 kb) (Fig. 6b). One band (3.6 kb) is the internal AMV *Eco*RI fragment which hybridizes lightly to HAX_4 because it contains only 200 base pairs homologous to this probe. The other band (4.4 kb), which is not detected by hybridization with EB3 (data not shown), probably represents an AMV provirus juncture fragment with chicken DNA adjacent to its 3' end. The presence of this presumptive juncture fragment was unexpected since the DNA was from total leukemic myeloblasts. Also, it is different from the 3' juncture fragment (2.0 kb) present in the AMV-λ clone 11A1-1. This 4.4-kb fragment was not previously detectable with viral probes because it comigrates with several *Eco*RI fragments of the same size which are internal to the helper myeloblastosis-associated virus and endogenous proviruses.

Five *amv*-specific bands (1.2, 1.9, 2.2, 2.9,

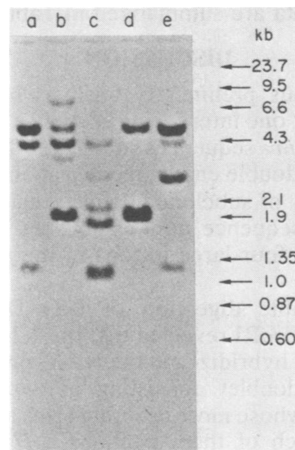

FIG. 6. Hybridization of restriction enzyme-digested leukemic DNA with HAX_4 probe. DNA from leukemic myeloblasts of chicken no. 21710 (18) was digested with either *Hin*dIII (a), *Eco*RI (b), *Hin*dIII and *Eco*RI (c), *Bam*HI and *Eco*RI (d), or *Hin*dIII and *Bam*HI (e). The DNA digests were electrophoresed in a 0.8% agarose gel, Southern blotted, and hybridized to ^{32}P-labeled HAX_4 DNA. A mixture of *Hin*dIII-digested λ DNA and *Hae*III-digested φX174 RF DNA was used as molecular size markers.

and 4.3 kb) were detected after hybridization of HAX_4 to C/E leukemic DNA digested with *Eco*RI + *Hin*dIII. Two fragments (1.2 and 2.9 kb) containing *amv* sequences were expected to be generated by *Eco*RI + *Hin*dIII digestion of the AMV provirus. Only the 2.9-kb band was detected (Fig. 6c) in addition to the pattern obtained with normal DNA, because the 1.2-kb band comigrated with the 1.2-kb band characteristic for *proto-amv* sequences (see above). The autoradiographic intensity of the 1.2-kb band generated from *Eco*RI + *Hin*dIII-digested DNA was in agreement with the presence of two *amv*-containing fragments.

If leukemic DNA digested with both *Bam*HI and *Eco*Ri was hybridized to the HAX_4 probe (Fig. 6d), three bands (5.4, 2.0, and 1.8 kb) were detected. The 2.0-kb band is broad and intense in the autoradiogram because it corresponds to two *proto-amv* fragments (see above). The 1.8-kb band corresponds to an *Eco*RI fragment generated from the AMV provirus, whereas the 5.4-kb band is a cellular fragment carrying *proto-amv* sequences.

The *Bam*HI + *Hin*dIII digestion of leukemic DNA (Fig. 6e) gave rise to four bands (5.2, 4.3, 2.8, and 1.3 kb) when hybridized with HAX_4 DNA. Only one band (2.8 kb) resulted from the double digestion of the integrated AMV provirus. The other bands corresponded to the *proto-amv*-containing fragments. As mentioned earlier, the 5.2-kb band consisted of two *proto-amv* fragments.

These data are summarized in Table 2.

DISCUSSION

A previous preliminary analysis had shown that at least one intervening sequence interrupts the *proto-amv* sequences in chicken DNA (12). The use of double enzymatic digestions together with probes of subcloned defined regions of the viral *amv* sequence now establishes that there are at least four large introns in the *proto-amv* sequences.

The double digestion of C/O DNA with *Hin*dIII + *Eco*RI revealed that the 2.2-kb *Eco*RI band which hybridizes to the HAX_4 probe (12) is in fact a doublet consisting of two adjacent fragments whose more accurate sizes are 2.0 and 2.1 kb. Each of them contains a *Hin*dIII site such that *Hin*dIII digestion generates a single 1.3-kb fragment (12). Hybridization with specific subclones representing different *amv* regions showed that the 2.0-kb *Eco*RI fragment contained *proto-amv* sequences homologous to the *amv* sequences located from the *Eco*RI site up to 150 to 200 base pairs beyond the *Sal*I site. These *Eco*RI and *Sal*I sites are separated by 1.45 kb in *proto-amv*, whereas they are separated by only 350 base pairs in *amv*. This reveals a new intron in *proto-amv*. The remaining portion of the 3′-proximal *amv* sequences are carried by the 2.1- and 8.7-kb *Eco*RI fragments.

The *proto-amv* sequences corresponding to the *amv* sequences located between the *Hae*II and *Eco*RI sites are present in a 2.2-kb *Hin*dIII-*Eco*RI fragment generated by *Hin*dIII + *Eco*RI double digestion. This fragment makes up the 3′ end of the 5.4-kb *Eco*RI fragment. The 5′-proximal 5.4-kb *Eco*RI fragment and the internal 2.0-kb *Eco*RI fragment which contain *proto-amv* sequences are not contiguous but are separated by a 0.95-kb *Eco*RI fragment which therefore defines the minimal length of an additional intron within the *proto-amv* sequences.

The location of the 3′-proximal 5.2-kb *Hin*dIII fragment which contains *proto-amv* sequences homologous to 0.2 kb of 3′-proximal *amv* sequences was established (i) by hybridization of single *Hin*dIII digests of C/O DNA with the λ111-derived E3 probe which contains the 3′-proximal *proto-amv* sequences (12) and (ii) by hybridization of double *Bam*HI + *Hin*dIII and *Hin*dIII + *Eco*RI digests with the *amv* subclone SX12.

Hybridization of single *Sma*I and double *Sma*I + *Eco*RI digests with *amv* subclones locates the *proto-amv* internal *Sma*I site at 1.1 kb outside the 3′ end of the 5.4-kb *Eco*RI fragment. This confirms that a 0.95-kb *Eco*RI fragment separates the 2.0- and 5.4-kb *Eco*RI fragments which contain *proto-amv* sequences. The location of the *Sma*I site within the 2.0-kb *Eco*RI fragment (12, 14) also suggests that the 5′-proximal *Eco*RI site of this fragment corresponds to the single *Eco*RI site present in *amv* (12, 14). It is unlikely that in the generation of the *amv* insert, splicing occurred between two *Eco*RI sites to regenerate an *Eco*RI site (5, 6, 15). Therefore, it is possible that a very small stretch (less than 30 nucleo-

TABLE 2. Restriction enzyme fragments from leukemic and normal DNAs which hybridized with HAX_4 probe

Enzymes	Fragments (kb) from the following type of DNA:	
	Leukemic	Normal
*Hin*dIII	1.3, 4.3,[a] 5.2	1.3, 5.2
*Eco*RI	2.0, 3.6,[a] 4.4,[a] 5.4, 8.7	2.0, 5.4, 8.7
*Hin*dIII + *Eco*RI	1.2,[b] 1.9, 2.2, 2.9,[a] 4.3	1.2, 1.9, 2.2, 4.3
*Bam*HI + *Eco*RI	1.8,[a] 2.0, 5.4	2.0, 5.4
*Hin*dIII + *Bam*HI	1.3, 2.8,[a] 4.3, 5.2	1.3, 4.3, 5.2

[a] Fragments originating from AMV provirus integrated in leukemic DNA.

[b] This band consists of at least one *proto-amv* and one *amv* fragment.

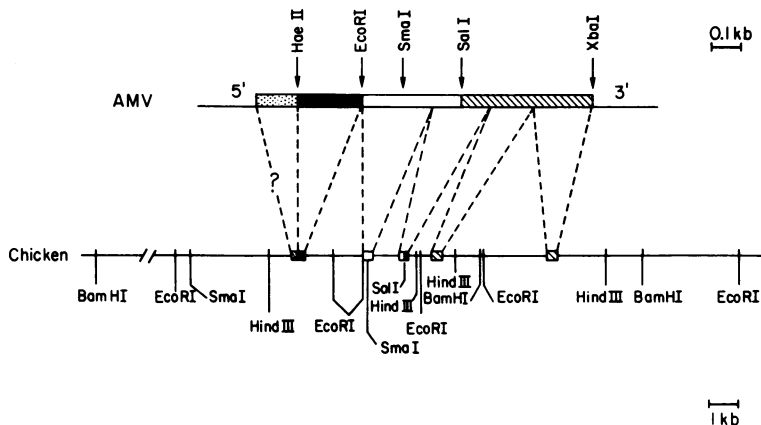

FIG. 7. Restriction endonuclease map of the chicken cellular DNA region containing *proto-amv* sequences. (Top) The *amv* insert of the AMV provirus has been subdivided into four regions corresponding to the hybridization probes used in this study. (Bottom) The recognition sites for various restriction enzymes have been mapped in the chicken DNA region containing the *proto-amv* sequences. The exact locations of the *proto-amv* sequences within the various DNA segments delineated by restriction enzyme sites are not known, except for the *amv* sequences located between the *Eco*RI and *Sal*I sites (see the text). The question mark at the 5′ end of *amv* indicates that we do not know whether this 5′ segment is contiguous with the *Hae*II site in chicken DNA. The size scales (in kilobases) for the viral (top) and cellular (bottom) sequences are different.

tides long) of *proto-amv* sequences, not long enough to be detected by hybridization, is located upstream of the corresponding *proto-amv* *Eco*RI site. These few nucleotides would permit splicing to generate the *amv* region within which the internal *Eco*RI site is located. The present results are summarized in Fig. 7, which shows a partial restriction map of the chicken DNA region containing *proto-amv* sequences. From our previous study (12) with the AMV *Kpn*I-*Xba*I probe, we know that the *amv* sequences located between the 5′ end of the *amv* insert and the *Hae*II site are present within the 2.2-kb *Hin*dIII-*Eco*RI fragment, but we do not know whether their 3′ end is contiguous with the *Hae*II site. The amount of *proto-amv* sequences carried by the 2.1- and 8.7-kb *Eco*RI fragments cannot be precisely determined. However, from the relative intensity of the 1.2- and 4.3-kb bands obtained in the double *Hin*dIII + *Eco*RI digest hybridized to HAX_4, it appears that the number of *proto-amv* sequences present on the 3′-proximal 4.3-kb fragment (i.e., within the 8.7-kb *Eco*RI fragment) is slightly greater than the number of *proto-amv* sequences present on the 1.2-kb fragment (i.e., within the 2.1-kb *Eco*RI fragment). A similar arrangement of *proto-amv* sequences occurred in all C/O and C/E chicken tissues examined, indicating their stability in development and differentiation.

Under the experimental approach used in this study, we detected the presence of four large introns which interrupt the *proto-amv* sequences, but we do not rule out the existence of additional smaller intervening sequences. They would not be detectable if they did not contain recognition sites for the restriction endonucleases we used. Therefore, the four introns detected in this study represent the minimum number. Also, as will be shown in our next paper on this subject (Perbal et al., submitted for publication), an additional intron was discovered with the same restriction enzymes as those used here if λ *proto-amv* DNA recombinant clones were analyzed instead of total chicken DNA.

The restriction endonuclease analysis performed on DNA isolated from leukemic myeloblasts did not reveal any major rearrangement of the cellular *proto-amv* sequences as a result of leukemogenesis. Characterization of chicken-λ DNA recombinants carrying *proto-amv* sequences isolated from leukemic cells confirms that the *proto-amv* arrangement in leukemic chicken DNA is similar to that in normal DNA. This implies that the leukemogenic effect of AMV may be entirely dependent upon transcription of the integrated AMV provirus. In support of this hypothesis, the *amv* transcript, which is shorter (2.5 versus 4.5 kb) than the RNA which is transcribed from the *proto-amv* sequences in hemopoietic chicken tissues (7, 10, 20; unpublished data), is very abundant in leukemic myeloblasts, whereas the *proto-amv* transcript is absent.

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