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Restriction Enzyme Sites on the Avian RNA Tumor Virus Genome

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Full-size single-stranded DNA transcripts of the avian RNA tumor virus genome were isolated from the products of the endogenous reaction of detergent-disrupted avian sarcoma virus particles. These transcripts were converted with *Escherichia coli* DNA polymerase I and ^{32}P-labeled nucleoside triphosphates into labeled double-stranded DNA. The latter DNA was used to map the sites of action of seven restriction enzymes (*Pvu* I, *Hpa* I, *Kpn* I, *Xba* I, *Eco*RI, *Hin*dIII, and *Xho* I) on the genome of three strains of avian sarcoma virus (Prague B, Prague C, and Bratislava 77).

In the endogenous reaction of detergent-disrupted RNA tumor viruses, the viral RNA is transcribed by the viral reverse transcriptase into DNA (1, 18). It has been shown that under some conditions of synthesis, a portion of DNA transcripts approach full genome length (2, 6, 8, 12). For the studies presented in this report, such potentially full-size DNA was isolated from the DNA products of the endogenous reaction of certain avian sarcoma viruses (ASV) and was converted, by the method of Summers (14) and Summers et al. (15), into ^{32}P-labeled double-stranded DNA. With this method, unlabeled single-stranded DNA is used as a template for *Escherichia coli* DNA polymerase I, in the presence of oligodeoxyribonucleotide primers and ^{32}P-labeled deoxynucleoside triphosphate precursors. With such labeled double-stranded DNA it has been possible to obtain information regarding the sites of action of restriction enzymes on the ASV genome. Maps are presented for seven enzymes: *Pvu* I, *Hpa* I, *Kpn* I, *Xba* I, *Eco*RI, *Hin*dIII, and *Xho* I.

MATERIALS AND METHODS

Viruses. The three strains of ASV used were the Prague strain of subgroups B and C and the Bratislava strain of subgroup C. These viruses were obtained from the laboratory of P. K. Vogt and were subsequently focus-cloned.

Viral DNA transcripts complementary to the viral genome. As previously described, viral DNA was transcribed in the endogenous reaction of detergent-disrupted virions (8, 9). [^{3}H]TTP (New England Nuclear Corp.) was present during the synthesis so that the DNA transcripts had a specific activity of 50 cpm/ng. About 10 μg of total labeled transcripts were extracted and subjected to sedimentation through an alkaline sucrose gradient. The DNA (about 5% of the total) with a sedimentation value consistent with genome-sized DNA was isolated (8, 9). Heteroduplex studies confirm that this DNA is not seriously contaminated with transcripts of transformation-defective genomes that have deletions in the *src* region (8, 9).

Conversion of single-stranded DNA transcripts into ^{32}P-labeled double-stranded DNA. The method of Summers (14) and Summers et al. (15) was used to convert 5 ng of single-stranded DNA into labeled double-stranded DNA. Synthesis was carried out in a siliconized glass tube (6 by 50 mm) for 1 h at 15°C in a volume of 10 μl containing 10 μM dGTP, 10 μM dCTP, 10 μM dTTP, 5 μM [α-^{32}P]dATP (350 Ci/mmol, New England Nuclear Corp.), 0.5 μg of template DNA per ml, 200 μg of calf thymus DNA primers per ml (14), and 50 μg of *E. coli* DNA polymerase I (a gift from L. Loeb) per ml. Synthesis was stopped with an excess of EDTA (0.01 M) and sodium dodecyl sulfate (0.5% wt/vol). After ethanol precipitation the product was subjected to electrophoresis on an agarose gel.

Gel electrophoresis. The horizontal and vertical slab gel apparatus designed by McDonell et al. (10) were obtained from the Aquebogue Machine and Repair Shop, Long Island, N.Y. Samples of not more than 20 μl were applied to horizontal gels (13 by 13 by 0.6 cm) of 1% agarose (Seachem). Further details are provided in the figure legends. Samples of not more than 20 μl were applied to vertical gels (13 by 13 by 0.125 cm) of 3 to 10% acrylamide, and electrophoresis was carried out at 4 V/cm for about 16 h. The electrophoresis buffer contained 0.04 M Tris-hydrochloride (pH 6.9), 0.02 M sodium acetate, and 0.001 M EDTA. As required, both agarose and acrylamide gels were dried onto paper (3 MM, Whatman) and subjected to autoradiography at −70°C with Kodak RP-5 and a Dupont Lightning Plus intensifying screen.

Elution of radioactive samples from agarose gels was carried out by the electrophoretic procedure of McDonell et al. (10). Briefly, the gel slice was sealed in dialysis tubing together with 0.4 ml of electrophoresis buffer cotaining 50 μg of yeast carrier RNA per

ml. After electrophoresis at 4°C for 3 h at 100 V, the electrode polarity was reversed for 30 s. The aqueous solution surrounding the gel contained at least 85% of the total radioactivity.

The molecular weight markers used in gel electrophoresis were *Hin*dIII fragments of phage lambda DNA (λcI857) and *Hae* III fragments of phage φX174 replicative form. Unlabeled lambda and the φX174 replicative form were obtained from New England Biolabs and Bethesda Research Laboratories, respectively, and then labeled in vitro with [^{32}P]dATP. Labeling was carried out in a siliconized glass tube (6 by 50 mm) for 30 min at 15°C in a volume of 10 μl containing: 0.1 M Tris-hydrochloride (pH 7.5), 0.02 M $MgCl_2$, 10 μM dCTP, 10 μM dGTP, 10 μM dTTP, 5 μM [^{32}P]dATP, 100 μg of unlabeled DNA per ml, and 50 μg of *E. coli* DNA polymerase I per ml. Synthesis was stopped with EDTA and sodium dodecyl sulfate followed by ethanol precipitation. The average specific activity of the labeled DNA was about 100 cpm/ng. The labeled DNAs were digested with *Hin*dIII and *Hae* III. The molecular weights of the *Hin*dIII fragments of phage lambda are those deduced relative to φX174 described by Cory and Adams (4). The exact molecular weight of the *Hae* III fragments of φX174 can be deduced from the data of Sanger et al. (13). Following Cory and Adams (4) we have used 618 as the average molecular weight of a nucleotide pair. Expressed in units of 10^6 daltons, the lambda *Hin*dIII fragments we have used are 14.7, 5.85, 4.09, 2.63, 1.40, and 1.22, and the φX174 *Hae* III fragments are 0.829, 0.666, 0.539, 0.375, 0.192, 0.170 (dimer), 0.145, 0.120, 0.0729, and 0.0445.

Restriction enzyme digestion. Digestion of ^{32}P-labeled ASV double-stranded DNA was carried out for at least 1 h at 37°C in a volume of 10 to 20 μl containing: 0.006 M Tris-hydrochloride (pH 7.4), 0.05 M NaCl, 0.006 M $MgCl_2$, 0.006 M mercaptoethanol, 4 U of enzyme, and not more than 5 ng of labeled DNA. Enzymes were obtained commercially, *Pvu* I, *Kpn* I, *Xho* I, *Xba* I, and *Hin*dIII were obtained from New England Biolab; *Hpa* I was obtained from Bethesda Research Laboratories; and *Eco*RI was obtained from Miles Laboratories. For digestion with *Pvu* I, the concentration of NaCl was increased to 0.15 M.

RESULTS

Viral DNA, labeled at low specific activity with [^{3}H]TMP, was synthesized in the endogenous reaction of detergent-disrupted PrC strain of ASV. The transcripts were extracted and sized on an alkaline sucrose gradient. About 5% of this DNA had a sedimentation value approximately that expected of genome-sized DNA (8, 9). About 5 ng of this DNA was converted to ^{32}P-labeled double-stranded DNA and analyzed by electrophoresis on a horizontal 1% agarose gel followed by autoradiography. Phage lambda DNA digested with *Hin*dIII provided molecular-weight markers (Fig. 1a). Relative to these markers the labeled ASV DNA (Fig. 1b) migrates as double-stranded DNA in the molecular-weight range of about 3×10^6 to 5.6×10^6. In fact, there is a relatively sharp cut-off on the high molecular-weight side at 5.6×10^6, a size comparable but slightly smaller than that of the linear double-stranded ASV DNA synthesized in infected cells. According to Varmus et al. (19), the molecular weight of the latter DNA is about 6×10^6.

[^{32}P]DNA equivalent to 5.6×10^6 daltons (Fig. 1b) was isolated by electroelution. This DNA has been used to obtain information regarding restriction enzyme sites on the viral genome. The DNA is relatively homogenous (Fig. 1c). It is sensitive to certain restriction enzymes as exemplified in Fig. 1d and e for *Eco*RI and *Xho* I, respectively. We have determined the size of restriction fragments obtained with each of the seven enzymes (Table 1). It can be seen that for each enzyme the total molecular weight of the fragment is about 5.6×10^6. Fragments of sizes as small as 0.35×10^6 daltons were measured on 1% agarose gels. Smaller fragments were measured on 3 to 10% gradient acrylamide gels, as exemplified in Fig. 2, with respect to the *Hae* III digest of phage φX174 replicative form (Fig. 2a). Figure 2b and c shows, for example, that *Eco*RI and *Hin*dIII, respectively, release relatively small restriction fragments.

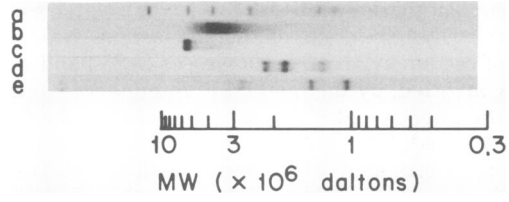

FIG. 1. *Agarose gel electrophoresis of double-stranded PrC ASV DNA. Samples were subject to electrophoresis for 16 h at 0.8 V/cm on a 1% agarose gel as described in the text. (a) ^{32}P-labeled phage lambda HindIII fragments. (b) Total ^{32}P-labeled double-stranded ASV DNA. (c) Re-electrophoresis of that DNA eluted from sample (b) that had a mobility equivalent to 5.6×10^6 daltons. (d) As in (c), but digested before electrophoresis with EcoRI. (e) As in (c), but digested before electrophoresis with Xho I.*

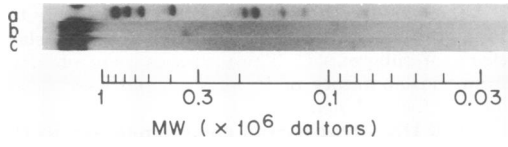

FIG. 2. *Acrylamide gel electrophoresis of double-stranded PrC ASV DNA. (a) ^{32}P-labeled phage φX174 replicative form Hae III fragments. (b) ^{32}P-labeled double-stranded ASV DNA with electrophoretic mobility of 5.6×10^6 daltons that was digested before electrophoresis with EcoRI. (c) As in (b), but digested with HindIII.*

Three standard approaches (11) have been used to obtain the order of the restriction fragments on the uncut DNA. First, we have examined digests in which two enzymes are used simultaneously. Second, we have purified by electrophoresis the three major *Eco*RI fragments and the three major *Xho* I fragments. Each of these was then submitted to digestion with a second enzyme. Such data are exemplified in Fig. 3 and summarized in Table 2. Third, we have made use of certain fragment associations observed in partial digests obtained with each enzyme. These three sources of information have allowed us to deduce the fragment maps presented in Fig. 4. The polarity of these maps is such that the 5'-terminus of the DNA strand complementary to the viral genome is shown on the right-hand side. This polarity could be defined because in a previous study we showed that the smallest *Eco*RI fragment, 0.095×10^6 daltons, is located at the 5'-terminus of this DNA (17).

DISCUSSION

In this study we have used the method of Summers (14) and Summers et al. (15) to convert apparently full-size single-stranded DNA transcripts of the ASV genome into ^{32}P-labeled double-stranded DNA. With such labeled DNA we have characterized the sites of action of seven restriction enzymes (Fig. 4). For a given restriction enzyme the amount of label per molecule of restriction fragment is not the same (Fig. 1d and e). This could be due largely to base composition

TABLE 1. *Molecular weight of fragments obtained by digestion with a single restriction enzyme of double-stranded PrC ASV DNA*

Fragment	Mol wt ($\times 10^6$)[a] obtained with:						
	Pvu I	*Hpa* I	*Kpn* I	*Xba* I	*Eco*RI	*Hin*dIII	*Xho* I
A	5.41	3.93	2.94	2.05	2.26	1.98	2.76
B	0.134	1.63	2.62	1.94	1.84	1.91	1.43
C				1.50	1.34	1.58	1.02
D					0.095	0.083	0.344
Total	5.55	5.56	5.56	5.49	5.54	5.55	5.55

[a] Molecular weights represent the average of two independent measurements.

TABLE 2. *Molecular weight of fragments obtained by digestion with two restriction enzymes of double-stranded PrC ASV DNA*[a]

Primary restriction enzyme	Mol wt ($\times 10^6$) of primary restriction enzyme fragment	Mol wt ($\times 10^6$) of fragments derived by secondary digestion of primary fragment with:						
		Pvu I	*Hpa* I	*Kpn* I	*Xba* I	*Eco*RI	*Hin*dIII	*Xho* I
*Eco*RI	2.26	2.26	1.99 0.27	1.58 0.68	1.62 0.64	2.26	1.91 0.27 0.083	1.75 0.51
	1.84	1.80 0.038	1.84	1.84	1.41 0.43	1.84	1.81 (0.03)	1.33 0.51
	1.34	1.34	1.34	1.34	1.34	1.34	1.34	1.00 0.34
Xho I	2.76	2.76	1.50 1.26	2.60 0.16	1.67 1.09	1.75 1.01	1.39 1.28 0.083	2.76
	1.43	1.30 0.134	1.43	1.43	1.43	1.33 0.095	1.43	1.43
	1.02	1.02	1.02	1.02	0.95 (0.07)	0.51 0.51	0.54 0.47	1.02

[a] The three major fragments derived by digestion with *Eco*RI and also by *Xho* I were purified by electrophoresis and subsequent electroelution. These six fragments were submitted to digestion with a second enzyme, and the resulting fragments were characterized by gel electrophoresis. Those fragments in parenthesis are predicted but have not been detected.

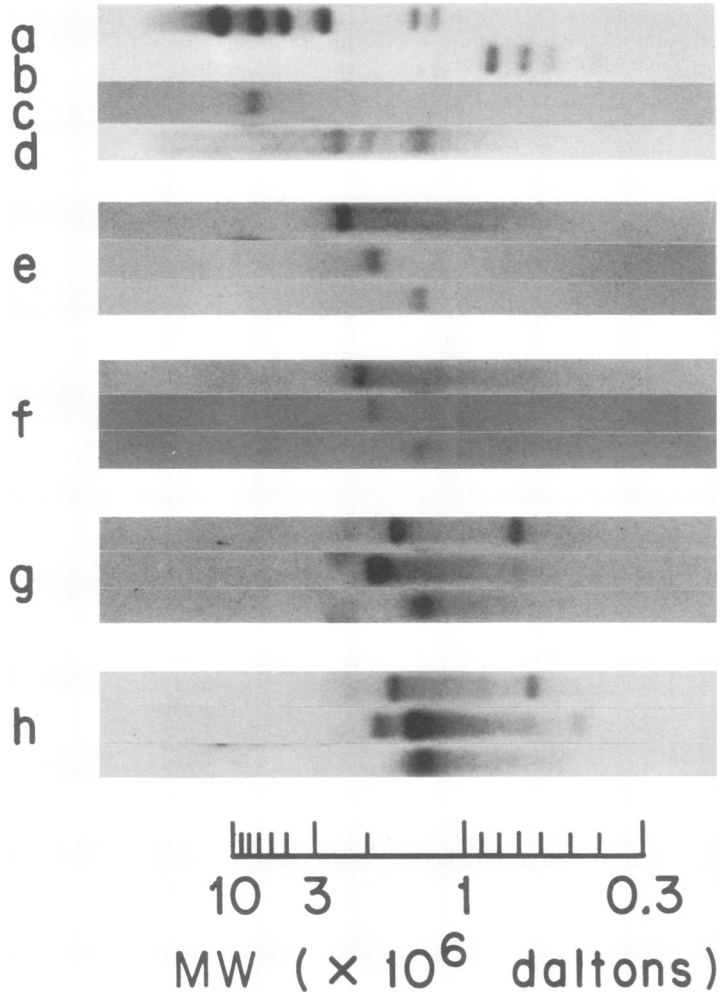

FIG. 3. *Agarose gel electrophoresis of recuts of isolated EcoRI fragments of double-stranded PrC ASV DNA. Samples were subject to electrophoresis for 2 h at 3.2 V/cm on a 1% agarose gel as described in the text. (a) ^{32}P-labeled phage lambda HindIII fragments. (b) ^{32}P-labeled phage φX174 replicative form Hae III fragments. (c) ^{32}P-labeled double-stranded ASV DNA with electrophoretic mobility equivalent to 5.6 × 10^6 daltons. (d) As in (c), but digested with EcoRI before electrophoresis. (e) The three major EcoRI fragments isolated from electrophoresis, such as (d). (f) As in (e), but recut with Hpa I. (g) As in (e), but recut with Kpn I. (h) As in (e), but recut with Xba I.*

differences between fragments since only one of the four triphosphates is labeled. In addition, fragments generated from the left-hand side of the genome (Fig. 4) occur in relatively lower molar amounts (for example, fragment D generated with *Xho* I). This could be due to premature termination of the complementary DNA transcript in the initial synthesis with reverse transcriptase and/or to incomplete replication of the 3′-terminus of that transcript in the subsequent synthesis with *E. coli* DNA polymerase I.

The restriction enzyme characterization presented here was carried out with the PrC strain of ASV DNA. With two exceptions, identical results were obtained with the PrB and B77 strains: for B77 DNA the *Hin*dIII site between fragments A and B is missing and for PrB there is an additional site for *Xho* I at 0.12 × 10^6 daltons from the left side of fragment C (Fig. 4).

Both the 5′- and 3′-termini of DNA complementary to the viral RNA genome have to be related to the termini of the viral RNA itself. As regards the 5′-terminus of the DNA, it has been shown that in the endogenous reaction of detergent-disrupted ASV, transcription of DNA is

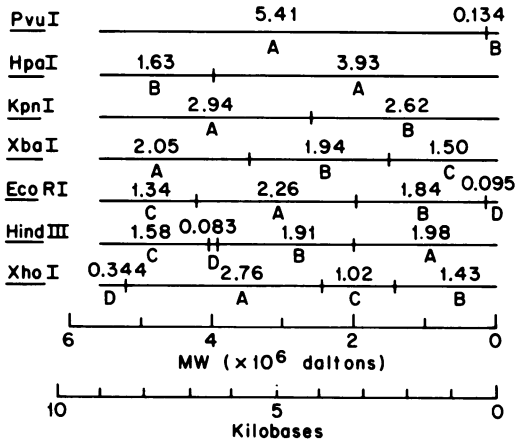

FIG. 4. *Ordering of restriction fragments obtained with double-stranded PrC ASV DNA. The letter code applied to the fragments is as listed in Table 1. The right-hand side represents the 5'-terminus of that DNA transcript complementary to the viral RNA.*

initiated on the 3'-terminus of a tRNA primer bound to the viral genome 101 nucleotides from the 5'-terminus of that genome (5, 16). Transcription immediately beyond 101 nucleotides is believed to occur by a jump that allows transcription of sequences at the 3'-terminus of the viral RNA, excluding the polyadenylic acid. Heteroduplex studies support this hypothesis (7). The jump could be facilitated by the terminally-redundant sequence observed immediately adjacent to polyadenylic acid (5). This sequence is presumably not transcribed (W. A. Haseltine, personal communication).

Where on the genome transcription of complementary DNA ultimately stops has not been determined, and there may be different results for transcription in vitro with respect to what occurs in vivo. For example, it could be that the 3'-terminus of the complementary DNA includes transcripts of the primer-binding site, another copy of the 101-nucleotides at the 5'-terminus of the RNA, and maybe even another copy of some sequences at the 3'-terminus of the genome.

Our immediate application of the restriction data reported here is to characterize those ASV DNA species, both integrated and unintegrated, that can be detected in infected avian cells. Similar studies are being carried out by P. Shank, J. Bishop, and H. Varmus. Recently, Collins and Parsons (3) published data on the *Eco*RI fragments of integrated ASV DNA. They observed the same three major fragments and deduced the same order. Relative to the present work, they claim an additional cleavage site at the left end of the integrated DNA (in the terminology of Fig. 4). One explanation of this apparent discrepancy is that certain in vivo ASV DNA species, both unintegrated and integrated, possess a terminal redundancy, relative to the genomic RNA, of about 0.25×10^6 daltons (J. Taylor, T. Hsu, J. Sabran, R. Guntaka, and W. Mason, unpublished data). The additional *Eco*RI site at the left end is actually a redundancy of the site at the right end.

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