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Genetics

Inverted repeat nucleotide sequences in the genomes of Marek disease virus and the herpesvirus of the turkey

(electron microscopy/single-stranded DNA/fold-back molecules)

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ABSTRACT **The DNAs of two herpesviruses, the oncogenic Marek disease virus and the serologically related herpesvirus of the turkey, were studied by electron microscopy. On the basis of fold-back molecules observed in single-stranded DNA from both viruses, structures have been derived for the overall nucleotide sequence arrangement in their genomes. Although differing in molecular weight, the genomes of Marek disease virus and turkey herpesvirus are both constructed according to the same plan—two regions of unique nucleotide sequence, each enclosed by inverted repeat sequences. The genome structure of these viruses therefore closely resembles that of herpes simplex virus rather than the biologically more similar herpesvirus Epstein–Barr virus, *H. saimiri*, and *H. ateles*.**

Marek disease virus (MDV), a herpesvirus, is the causative agent of Marek disease, a malignant lymphoma of chickens (for review, see refs. 1–3). From tumors of diseased animals, continuous lymphoblastoid T-cell lines can be established (4, 5) and these have been shown to harbor the MDV genome in a latent form (6–8). A serologically related herpesvirus of the turkey (HVT) (9–11) produces "microlymphomas" in its natural host and in chickens and, being essentially apathogenic for both, has been used as a vaccine against Marek disease (12).

A somewhat similar "lymphotropic" biology is shared by several other members of the herpesvirus group. In humans, the Epstein–Barr virus (EBV) causes heterophile-positive infectious mononucleosis (for review, see refs. 13 and 14) and is considered to be a cofactor in two neoplastic diseases—Burkitt lymphoma and nasopharyngeal carcinoma (15–17). In new world monkeys, the herpesviruses *H. saimiri* and *H. ateles* induce highly lethal lymphoproliferative diseases in heterologous hosts (for review, see refs. 18 and 19). EBV, *H. saimiri*, and *H. ateles*, are, like MDV, able to enter into a stable proliferation-stimulating association with lymphoid cells [B cells in the case of EBV (16), T cells in the case of *H. saimiri* and *H. ateles* (19)] in which the viral genome is present as a plasmid-like circular DNA molecule (20, 21). Because of such similarities, MDV, HVT, EBV, *H. saimiri*, and *H. ateles* have been grouped together in the *Herpesviridae* subfamily *Gammaherpesvirinae* (22).

EBV, *H. saimiri*, and *H. ateles* also share certain peculiarities in the arrangement of nucleotide sequences within their DNAs. All have genomes containing direct tandem repeats of short (M_r, 0.4–2.0 × 10^6) nucleotide sequences; *H. saimiri* and *H. ateles* carry a single sequence that is present in multiple copies at both extremities of the genome (23, 24) and EBV carries two nonhomologous sequences, one repeated at the extremities and a second at the interior of the genome (25–27). This sort of sequence arrangement stands in contrast to a second type found in other herpesviruses (subfamily *Alphaherpesvirinae*, ref. 22) that have a nonlymphotropic biology, such as herpes simplex virus (HSV), pseudorabies virus, equine abortion virus, and bovine mammillitis virus. A characteristic feature of these genomes is the presence of extensive (M_r, 6–10 × 10^6) inverted repeat nucleotide sequences enclosing either one (pseudorabies virus, refs. 28 and 29; equine abortion virus, unpublished) or two (HSV, refs. 30–33; bovine mammillitis virus, ref. 34) regions of unique base sequence.

In light of the biological properties outlined above, one might expect the genomes of MDV and HVT to resemble those of EBV, *H. saimiri*, and *H. ateles*. The results of the study reported here do not, however, fulfill this expectation and show instead that the genomes of MDV and HVT contain inverted repeat sequences in the configuration found in HSV and BMV. A preliminary report of this work has been presented elsewhere.‡

MATERIALS AND METHODS

Virus Strains, Cells, Virus Growth and Purification, and DNA Preparation. These have been described (8). Four strains of MDV were used in this study: the pathogenic GA and JM strains, the apathogenic Cal-1 strain, and the attenuated CV1-988 strain. The HVT strain used was PBTHV1.

Electron Microscopy. The spreading of DNA was carried out with a 50% formamide hyperphase and a 17% formamide hypophase according to standard procedures (35). Single-stranded DNA was prepared by alkaline denaturation and reneutralization (36) and subsequently dialyzed against 0.1 M Tris·HCl, pH 8.5/0.1 M EDTA in 50% formamide. In some experiments, the neutralized preparations were adjusted to 1 M NaCl and incubated at 22°C for 1 hr before dialysis to enhance self-hybridization, although this procedure was generally not necessary. Double-stranded PM2 DNA [M_r, 6.4 × 10^6 (37)] and single-stranded φX174 DNA [M_r, 1.7 × 10^6 (38)] were included in the spreading solution as internal molecular weight standards. Grids were examined and photographed with a Siemens model 1A electron microscope. Contour lengths were measured from enlarged positive prints with a digitizer board interfaced to a Hewlett–Packard model 9820A computer. All length measurements were referred to PM2 and φX174 DNA molecules in the same field.

RESULTS

Our initial electron microscopic observations of denatured DNA from the four MDV strains and the HVT strain showed consid-

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Abbreviations: MDV, Marek disease virus; HVT, herpesvirus of the turkey; EBV, Epstein–Barr virus; HSV, herpes simplex virus.

‡ Cebrian, J., Kaschka-Dierich, C., Berthelot, N. & Sheldrick, P., Fourth Herpesvirus Workshop, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, August 1979.

erable size heterogeneity in the populations of single-stranded molecules. Molecular weights (deduced by comparison with single-stranded ϕX174 DNA included as internal standard) varied from $\approx 5 \times 10^6$ to $\approx 60 \times 10^6$; molecules at the extremes of this range were infrequently encountered relative to those near the midpoint. Such size heterogeneity was not totally unexpected. It is known that the double-stranded DNA isolated from the virions of many herpesviruses—including MDV and HVT (39, 40)—is subject to extensive fragmentation upon denaturation, presumably as a result of preexisting interruptions in the single strands (for review, see ref. 41).

Approximately 50% of the molecules in the preparations were in the form of looped fold-back structures, and 92 examples were selected for further study. Of these, 14 were double looped; two examples, one from MDV and the other from HVT, are shown in Fig. 1 *A* and *B*. Both loops are single stranded and of unequal size. In terms of the ϕX174 DNA internal standard, the molecular weights of the small (U_S) and large (U_L) loops, respectively, were estimated to be $3.7–3.9 \times 10^6$ and $36–41 \times 10^6$ for MDV and $\approx 2.8 \times 10^6$ and $\approx 35 \times 10^6$ for HVT. The two loops in these structures are joined by a "stem" of double-stranded DNA, whose molecular weight (estimated from comparison with PM2 DNA) is $\approx 17 \times 10^6$ for MDV and $\approx 13 \times 10^6$ for HVT. The total molecular weight of this structural form is therefore $\approx 60 \times 10^6$ for MDV and $\approx 51 \times 10^6$ for HVT. These values are in agreement with the expected molecular weights of intact single DNA strands from these genomes; the native DNA of MDV has been assigned a value of $110–120 \times 10^6$ (39, 40) and the DNA of HVT is "slightly less" (40).

Our immediate interpretation of the double-looped molecules was that, as with HSV (30–33) and bovine mammillitis virus (34), the genomes of MDV and HVT are constructed of a long region (U_L) and a short region (U_S) of nonrepeated base sequences, each flanked by inverted repeat sequences. The double-stranded stem would therefore correspond to the total extent of the inverted repeats [(IR_L/TR_L) + (IR_S/TR_S)]; for MDV, this is $\approx 34 \times 10^6$, and for HVT, this is $\approx 26 \times 10^6$. This view was supported by the remaining fold-back molecules, which were single looped. In the majority (63 of 78) of these, the single-stranded loop was the same size as the small loop in the double-looped molecules from the same virus and thus clearly appeared to correspond to region U_S (Fig. 1*C*). The du-

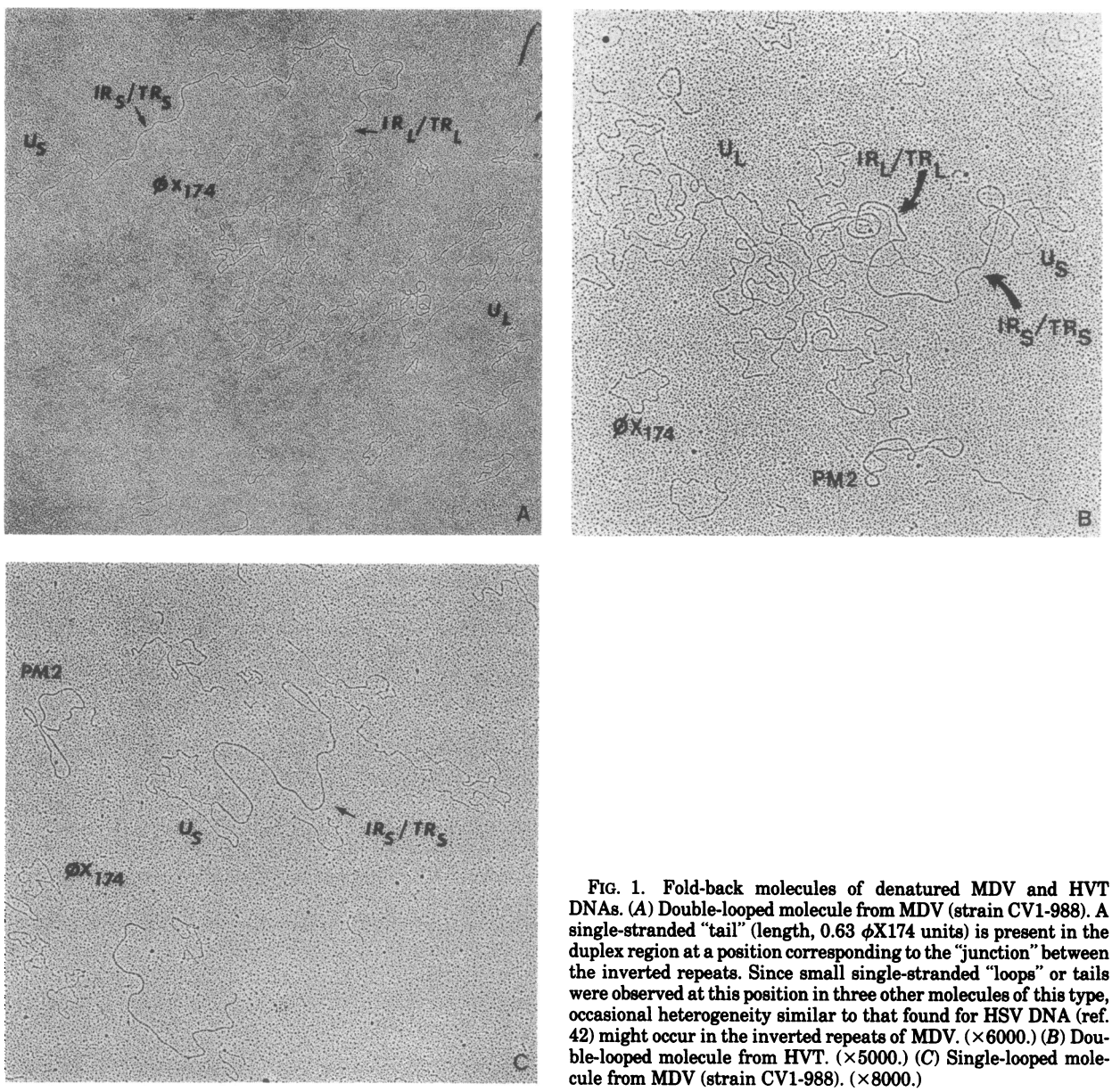


FIG. 1. Fold-back molecules of denatured MDV and HVT DNAs. (*A*) Double-looped molecule from MDV (strain CV1-988). A single-stranded "tail" (length, 0.63 ϕX174 units) is present in the duplex region at a position corresponding to the "junction" between the inverted repeats. Since small single-stranded "loops" or tails were observed at this position in three other molecules of this type, occasional heterogeneity similar to that found for HSV DNA (ref. 42) might occur in the inverted repeats of MDV. (×6000.) (*B*) Double-looped molecule from HVT. (×5000.) (*C*) Single-looped molecule from MDV (strain CV1-988). (×8000.)

Table 1. Contour length measurements and molecular weights of fold-back molecules in single-stranded MDV and HVT DNAs

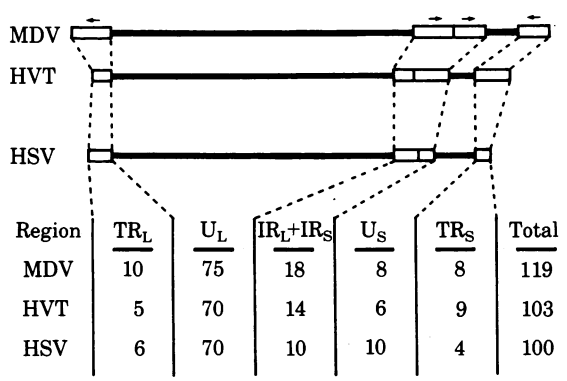
Virus	U_L region			U_S region			IR_S/TR_S region			$(IR_L/TR_L) + (IR_S/TR_S)$		
	n	ϕX174 units	M_r ($\times 10^{-6}$)	n	ϕX174 units	M_r ($\times 10^{-6}$)	n	PM2 units	M_r ($\times 10^{-6}$)	n	PM2 units	M_r ($\times 10^{-6}$)
MDV												
GA	4	21.8 ± 0.4	37	24	2.21 ± 0.10	3.7	16	1.29 ± 0.12	8.2	3	2.73 ± 0.10	17.5
JM	1	21.2	36	9	2.30 ± 0.09	3.9	7	1.21 ± 0.08	7.7		ND	ND
Cal-1	3	23.9 ± 1.0	41	11	2.25 ± 0.13	3.8	9	1.29 ± 0.06	8.2	2	2.59 ± 0.13	16.6
CV1-988	11	21.1 ± 1.3	36	20	2.18 ± 0.09	3.7	15	1.22 ± 0.15	7.8	5	2.73 ± 0.16	17.5
HVT (PB-THVI)	11	20.7 ± 0.7	35	13	1.66 ± 0.14	2.8	6	1.37 ± 0.05	8.8	4	2.10 ± 0.20	13.4

Length measurements are mean ± SD. Approximate molecular weights are calculated from 1 ϕX174 unit = 1.7×10^6 (ref. 38) and 1 PM2 unit = 6.4×10^6 (ref. 37). ND, not determined.

plex stem associated with the loop, however, was significantly shorter than the stem of double-looped molecules; for MDV, the length corresponds to a molecular weight of $7.7–8.2 \times 10^6$, and for HVT, it is $\approx 8.8 \times 10^6$. The stem arises through the self-hybridization of inverted repeats about region U_S and therefore stem length provides a direct measure of their size: $7.7–8.2 \times 10^6$ for each arm of IR_S/TR_S of MDV and $\approx 8.8 \times 10^6$ for the corresponding arms of HVT.

An additional feature of these molecules is the presence of a linear single-stranded "tail" extending from the end of the stem opposite to that bearing the loop (Fig. 1C). The lengths of the tails were extremely heterogeneous, ranging from barely visible (<200 bases) to nearly the length expected for region U_L and its associated inverted repeats (equivalent M_r, $40–50 \times 10^6$). As a result of this heterogeneity (undoubtedly due to the single-strand breakage previously alluded to), the tails were not used in estimating the size of region U_L.

A second type of single-looped molecule was, however, useful for this purpose. In this form (not shown), of which 15 examples were observed, the loop is very large and in fact was found to correspond in size to the large loop of double-looped molecules. Besides the loop, a duplex stem and an associated single-stranded tail of variable length were also present. The stem should reflect the fold-back of the inverted repeats (IR_L/TR_L) enclosing region U_L, and in seven molecules, the stem lengths fitted well with the size of these regions predicted by simply subtracting the value of the IR_S/TR_S region from the length of the stem in double-looped forms. The relevant measurements, converted into molecular weights, are MDV (CV1-988), 7.7, 9.0, and 10.4×10^6; MDV (JM), 8.8×10^6; HVT, 4.3, 4.8, and 5.7×10^6. The remaining molecules carried considerably shorter stems, again presumably reflecting single-strand breakage.



Region	TR_L	U_L	IR_L+IR_S	U_S	TR_S	Total
MDV	10	75	18	8	8	119
HVT	5	70	14	6	9	103
HSV	6	70	10	10	4	100

FIG. 2. Comparison of genome structures of MDV, HVT, and HSV. The molecular weights for the various regions of MDV and HVT are from Table 1 and that of HSV is from refs. 30–33. The molecular weights for regions U_L and U_S, which are for single-stranded DNA in Table 1, have been converted to values for the duplex form. Results are $\times 10^{-6}$.

A summary of contour length measurements for the various genomic regions and the molecular weights calculated from them is given in Table 1. Where possible, measurements obtained from each of the three molecular forms discussed above were combined to determine the appropriate values for a given region. Thus, for example, the data concerning region U_L of MDV strain CV1-988 were derived from five double-looped molecules and six single (large)-looped molecules. The present results have been used to construct schematic representations of the MDV and HVT genomes, and these, together with a similar one of the HSV genome for comparison, are illustrated in Fig. 2.

DISCUSSION

The various looped structures formed by the DNA strands of MDV and HVT leave little doubt that the genomes of these viruses not only resemble each other but also possess the basic sequence arrangement found in the genome of HSV (30–33). What is less certain is the accuracy of the size estimates for regions U_L and U_S, which have been derived by reference to single-stranded ϕX174 DNA (Table 1). In similar measurements for HSV, it was necessary to introduce a factor to correct for an apparent shrinkage of single strands (30), presumably a result of the high G+C content (68%) of HSV DNA relative to that of ϕX174 DNA (45%; ref. 38). This has not been done here, principally because the G+C contents of MDV and HVT DNAs are also $\approx$45% (39, 40), and so confirmation of the present values will be necessary. However, our estimates of total genome molecular weights are not discordant with those previously published by others.

In calculating the sizes of the various genomic regions shown in Fig. 2, we have assumed no strain differences and have combined the measurements obtained from the four MDV strains. Apart from the limited number of examples of each strain and the dispersion of the measurements (Table 1), this appears to be justified by the absence of major differences in restriction endonuclease digestion patterns of these DNAs (8, 40). With respect to the measurements comparing MDV and HVT, the differences in region size, with the possible exception of U_L, lie within the range of experimental error (Table 1). The total extent of the inverted repeats is greater in MDV (36×10^6) compared with HVT (28×10^6), as is the size of region U_S and, with somewhat less certainty, the size of region U_L. A clearer demonstration of the dissimilarity of these genomes, albeit at a different level, has already been provided by the findings that their DNAs fail to cross-hybridize and show quite different restriction profiles (8, 40).

If parallels in genome structure extend beyond the presence of inverted repeats, then in MDV and HVT one might expect to see inversion of regions U_L and U_S, which occurs in the genome of HSV (32, 34, 43, 44). There is at present no direct evidence of this, but inversion has been considered among several possible explanations for the appearance of fragments that have unequal molar ratios in restriction digests of MDV and HVT DNAs (8, 40). Another important aspect of the HSV genome structure is the presence of direct terminal repeats (30, 45), which have been shown to be a subset of the inverted repeats (31, 42, 46). Although we have not addressed this question for MDV and HVT, Tanaka *et al.* (7) have reported finding circular MDV DNA in lymphoblastoid cells, which would be an element in favor of such a possibility.

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