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Electron Microscopic Study of Equine Herpesvirus Type 1 DNA

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Electron microscopic studies of equine herpesvirus DNA revealed that single strands that were allowed to reanneal formed single-stranded loops with double-stranded stems only at one end of the molecule. These observations support restriction enzyme analyses which indicate that the 92-megadalton DNA molecule exists as a long region of unique sequences covalently linked to a short region. The short region is comprised of an internal unique sequence, which forms the loop during reannealing of single strands, and two terminal inverted repeat sequences that bracket the unique sequence and form the double-stranded stem structure observed upon reannealing of single strands. Measurements of the unique sequence and terminal inverted repeat subgenomic sequences indicate a size of 6.4 megadaltons for each and thus fix the size of the short region at approximately 19.2 megadaltons.

Equine herpesviruses have proved to be suitable for the study of the varied biological consequences of herpesvirus infection as model systems have been developed to investigate cytocidal infection in cell culture and animals (8), persistent infection (9, 13; S. A. Dauenhauer, R. A. Robinson, and D. J. O'Callaghan, J. Gen. Virol. in press), biochemical transformation (1), and oncogenic transformation (9–13). To elucidate which viral genes and gene products are associated with each of these aspects of herpesvirus infection, it is necessary that a detailed understanding of the overall genomic structure and arrangement of viral DNA sequences be obtained. Recently, we defined the physical structure of the equine herpesvirus type 1 (EHV-1) genome by restriction enzyme mapping techniques and proposed that this herpesviral DNA is a 92-megadalton (Md) molecule that is comprised of a 71.6-Md long region and a 20.4-Md short (S) region which contains inverted repeat sequences that bracket a unique sequence (Us) segment (6). Calculations of the size of the inverted repeat sequences and Us segment were based on a series of deductions made from data concerning the location of restriction enzyme sites within the S region. These calculations indicated that both the repeats and Us sequences were approximately 6 Md. Our recent finding that the genome of EHV-1 defective particles with the capacity to mediate persistent infection is comprised of reiterated copies of S-region sequences (9) prompted us to employ electron microscopic methods to examine and measure the Us and repeat segments of the S region of the standard genome.

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EHV-1 was propagated in L-M cell suspension cultures, and viral DNA was extracted from purified virions as described previously (6). Viral DNA (1.5 μg/ml), suspended in 0.2 M Tris-hydrochloride (pH 8.5)–0.001 M EDTA buffer, was denatured by heating at 60°C for 10 min in 80% formamide (vol/vol, recrystallized). Buffer (0.2 M Tris-hydrochloride, 0.02 M EDTA, pH 8.5) was added to reduce the formamide concentration to 66%, and the DNA was then allowed to self-anneal at 25°C for 2 h. After the self-annealing step, fd phage and simian virus 40 form II DNAs were added as single-strand and double-strand size markers, respectively. Finally, cytochrome *c* was added to a concentration of 0.3 mg/ml. The DNA was picked up from a 10% formamide hypophase (4) with Parlodion-coated 200-mesh copper grids. The grids were stained with uranyl acetate, rinsed with isopentane, and rotary shadowed with gold-palladium in an Edwards evaporator. Grids were examined in a Zeiss EM 10A electron microscope and photographed at a magnification of ×9,800. Negative photographs of individual self-annealed molecules were projected onto a blackboard with a lantern slide projector, and the DNA contour lengths were determined with a Keuffel and Esser map measure. Double-stranded molecular weights for single- and double-stranded regions of self-annealed EHV-1 molecules were

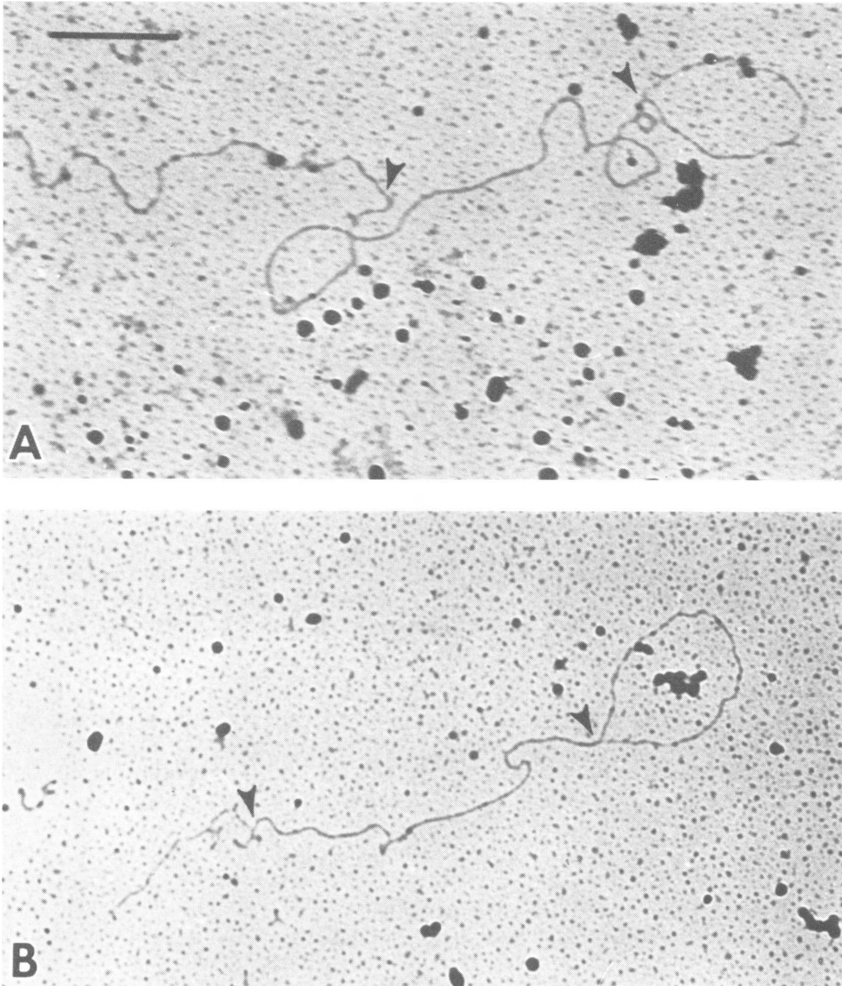

FIG. 1. Electron micrographs of self-annealed EHV-1 DNA molecules. The arrows delineate the double-stranded region corresponding to the inverted repeat. Bar = 0.5 μm.

calculated from the ratio of the observed EHV-1 DNA lengths and marker DNA lengths based on sizes of 2.11 Md for fd DNA (2) and 3.4 Md for simian virus 40 DNA (5).

Figure 1 shows electron micrographs of sections of EHV-1 self-annealed single strands. Panels A and B illustrate structures containing a single-strand loop contiguous to a double-strand region which terminates in a single-strand stretch. The double-strand region of the molecule in Fig. 1B is shorter than that in the structure in Fig. 1A, and the single-strand tail is quite short, indicating that the molecule contained single-strand interruptions at these points. Many (≥70%) of the molecules observed appeared to have random single-strand breaks either in the double- or single-strand regions or in both. This result is in keeping with the fact that alkaline sucrose gradient sedimentation of EHV-1 DNA indicates the presence of numerous, apparently random, single-strand breaks in the DNA (D. J. O'Callaghan, G. A. Gentry, and C. C. Randall, *in* B. Roizman, ed., *Comprehensive Virology*, Series 2, in press). Molecules which contained double loops and molecules which contained loops less than one or greater than two fd DNA contour lengths were not observed.

Histograms of the double-strand molecular weights calculated from the contour lengths of the single-strand loops which correspond to the Us region of the DNA and the linear double-strand regions which correspond to the inverted repeats are shown in Fig. 2. The average size from this analysis of the segment of the S region bounded by the repeats is 6.4 ± 1.1 Md. This value is in agreement with the value of approximately 6 Md for the Us segment determined by restriction enzyme analysis of the EHV-1 genome (6). The histogram of the measurements

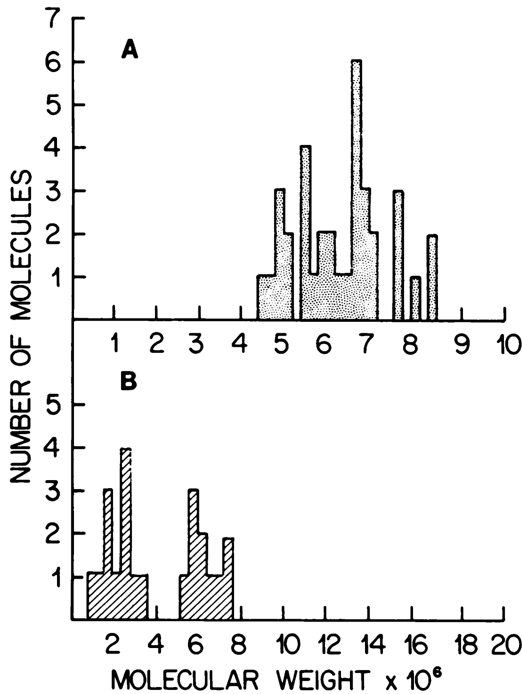

FIG. 2. Histograms of the double-strand molecular weight of the loop (A) and double-strand repeat regions (B) of self-annealed EHV-1 DNA. Note the biphasic nature of the repeat histogram.

of the terminal repeat segment shows two populations with average sizes of 2.4 ± 0.77 and 6.4 ± 0.89 Md. The size of the larger population is similar to the value of approximately 6 Md for the repeat size which has been determined by restriction enzyme analysis (6). Intramolecular annealing experiments were also carried out on a second, separate isolate of EHV-1 DNA. Measurements of 31 of the resulting structures yielded a value of 6.5 ± 1.1 Md for the Us region. Sizes for the repeat region once again fell into two groups, with means of 2.4 ± 0.62 and 6.2 ± 0.76 Md. The second set of measurements is in good agreement with the first and indicates that the observed features are indeed indicative of the structure of the EHV-1 genome.

Two possible explanations arise for the two values obtained for the size of the repeat region: (i) there exist two populations of EHV-1 DNA molecules with different-sized repeat regions or (ii) there exists a relatively localized single-strand break(s) within the terminal repeat. Since the molecules with differing repeat sizes appear to be present in approximately equal amounts (Fig. 2), the first explanation would require the presence of two sets of submolar restriction fragments in restriction enzyme digests of the DNA. This is not the case. Only a single set of 0.5 M fragments corresponding to a repeat region of approximately 6 Md has been observed during restriction mapping of the genome (6). The value of the 2.4 Md for the shorter population of repeats, therefore, probably indicates the existence of a single-strand break or breaks some 3.6 to 4.0 Md in from the end of the terminal repeat.

Thus, electron microscopic measurements reveal that the S region is comprised of two 6.4-Md inverted repeats and a 6.4-Md Us segment, which would indicate that the S region is 19.2 Md; this supports the 20.4-Md calculation obtained by restriction enzyme analyses (6). Contour lengths of 20 native, well-extended EHV-1 DNA molecules with simian virus 40 form II DNA as a size marker gave a size for the entire genome of 90.6 ± 7.8 Md, which is in good agreement with the value of 92 Md obtained by restriction enzyme mapping (6) and physicochemical measurements (14).

The work presented here shows that EHV-1 DNA has an internal structure similar to that of pseudorabies virus (3, 7, 15) and varicella-zoster virus (S. E. Straus, J. Owens, W. E. Ruyechan, H. A. Takiff, T. A. Casey, G. F. Vande Woude, and J. Hay, Proc. Natl. Acad. Sci. U.S.A., in press) in that the sequences from one terminus are repeated internally in an inverted form and these repeats bound a short stretch of unique DNA. These studies confirm earlier, unpublished observations concerning the structure of EHV-1 DNA (P. Sheldrick, personal communication) and corroborate recent restriction enzyme mapping of the EHV-1 genome (6). Lastly, it should be mentioned that electron microscopic and restriction enzyme mapping studies in our laboratories have shown that EHV-3 DNA has a structure similar to that of EHV-1 DNA and is comprised of a fixed long region and an S region that can invert due to the presence of inverted repeat sequences (S. S. Atherton, D. C. Sullivan, S. A. Dauenhauer, W. T. Ruyechan, and D. J. O'Callaghan, Virology, in press).

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LITERATURE CITED

1. **Allen, G. P., J. J. McGowan, G. A. Gentry, and C. C. Randall.** 1978. Biochemical transformation of deoxythymidine kinase-deficient mouse cells with ultraviolet-irradiated equine herpesvirus type 1. J. Virol. **28**:361-367.
2. **Beck, E., R. Sommer, E. Auerswald, C. Kurz, B. Zink, G. Osterburg, H. Schaller, K. Sugimoto, H. Sugiaski, T.**

Okamoto, and M. Takanami. 1978. Nucleotide sequence of bacteriophage fd DNA. Nucleic Acids Res. **5**:4495–4503.

3. **Ben-Porat, T., F. J. Rixon, and M. Blankenship.** 1979. Analysis of the structure of the genome of pseudorabies virus. Virology **95**:285–294.
4. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. **21**:413–428.
5. **Fiers, W., R. Contreras, G. Haegman, R. Rogiers, A. Van de Voorde, H. Han Heuverswyn, J. Van Hereweghe, G. Volckaert, and M. Ysebaert.** 1978. Complete nucleotide sequence of SV40 DNA. Nature (London) **273**:113–120.
6. **Henry, B. E., R. A. Robinson, S. A. Dauenhauer, S. S. Atherton, G. S. Hayward, and D. J. O'Callaghan.** 1981. Structure of the genome of equine herpesvirus type I. Virology **115**:97–114.
7. **Jean, J. H., M. L. Blankenship, and T. Ben-Porat.** 1977. Replication of herpesvirus DNA. I. Electron microscopic analysis of replicative structures. Virology **79**:281–291.
8. **O'Callaghan, D. J., G. P. Allen, and C. C. Randall.** 1978. Structure and replication of equine herpesviruses. p. 1–32. *In* J. T. Bryans and H. Gerber (ed.), Equine infectious disease IV. Veterinary Pub., Princeton, N.J.
9. **O'Callaghan, D. J., B. E. Henry, J. H. Wharton, S. A. Dauenhauer, R. B. Vance, J. Staczek, S. S. Atherton, and R. A. Robinson.** 1981. Equine herpesviruses: biochemical studies on genomic structure, DI particles, oncogenic transformation, and persistent infection, p. 387–418. *In* Y. Becker (ed.), Developments in molecular virology, vol. 1. Herpesvirus DNA. Nijhoff, The Hague.
10. **Robinson, R. A., B. E. Henry, R. G. Duff, and D. J. O'Callaghan.** 1980. Oncogenic transformation by equine herpesviruses (EHV). I. Properties of hamster embryo cells transformed by ultraviolet-irradiated EHV-1. Virology **101**:335–362.
11. **Robinson, R. A., and D. J. O'Callaghan.** 1981. The organization of integrated herpesviruses DNA sequences in equine herpesvirus type 1 transformed and tumor cell lines, p. 419–436. *In* Y. Becker (ed.), Developments in molecular virology, vol. 1. Herpesvirus DNA. Nijhoff, The Hague.
12. **Robinson, R. A., P. W. Tucker, S. A. Dauenhauer, and D. J. O'Callaghan.** 1981. Molecular cloning of equine herpesvirus type 1 DNA: analysis of standard and defective viral genomes and viral sequences in oncogenically transformed cells. Proc. Natl. Acad. Sci. U.S.A. **78**:6684–6688.
13. **Robinson, R. A., R. B. Vance, and D. J. O'Callaghan.** 1980. Oncogenic transformation by equine herpesvirus (EHV). II. Coestablishment of persistent infection and oncogenic transformation of hamster embryo cells by equine herpesvirus type 1 preparations enriched for defective interfering particles. J. Virol. **36**:204–219.
14. **Soehner, R. L., G. A. Gentry, and C. C. Randall.** 1965. Some physico-chemical characteristics of equine abortion virus nucleic acid. Virology **26**:394–405.
15. **Stevely, W. S.** 1977. Inverted repetition in the chromosome of pseudorabies virus. J. Virol. **22**:232–234.