

Renaturation of Complementary DNA Strands by Herpes Simplex Virus Type 1 ICP8

REBECCA ELLIS DUTCH AND I. R. LEHMAN*

Department of Biochemistry, Stanford University School of Medicine Center,
Stanford, California 94305-5307

Received 21 June 1993/Accepted 27 August 1993

ICP8, the major single-stranded DNA-binding protein of herpes simplex virus type 1, promotes renaturation of complementary single strands of DNA. This reaction is ATP independent but requires Mg^{2+} . The activity is maximal at pH 7.6 and 80 mM NaCl. The major product of the reaction is double-stranded DNA, and no evidence of large DNA networks is seen. The reaction occurs at subsaturating concentrations of ICP8 but reaches maximal levels with saturating concentrations of ICP8. Finally, the renaturation reaction is second order with respect to DNA concentration. The ability of ICP8 to promote the renaturation of complementary single strands suggests a role for ICP8 in the high level of recombination seen in cells infected with herpes simplex virus type 1.

ICP8, a 128-kDa zinc metalloprotein (19), is the major single-stranded DNA (ssDNA)-binding protein of herpes simplex virus type 1 (HSV-1) and appears to play several important roles in HSV-1 infection. Genetic analyses have shown that mutations in UL29, the gene encoding ICP8 (36), produce mutants defective in viral DNA replication (10, 26, 46) and that ICP8 is one of the seven HSV-1-encoded polypeptides necessary for viral DNA replication (32, 40, 49). ICP8 plays a role in the control of HSV-1 gene expression (16–18) and appears to act in localizing replication proteins to appropriate sites in the infected cell (7, 13, 35, 48).

In the absence of DNA, ICP8 polymerizes to form long helical filaments. It binds ssDNA preferentially (25, 38, 39), and the binding is both tight and cooperative (25, 38). ICP8 has been found to lower the melting temperature of synthetic polynucleotides (34, 44), and it can destabilize partial DNA duplexes (3). The stoichiometry of binding has been estimated by nuclease protection to be one ICP8 per 12 nucleotides (31) or per 22 nucleotides (22), with the variation likely due to different preparations of ICP8. A binding-site size of one ICP8 per 40 nucleotides has been estimated by electron microscopy. These studies have also shown that ICP8 binds ssDNA in regular repeating units, holding the DNA in an extended conformation (38, 39). ICP8 stimulates the activity of three of the HSV-1 replication enzymes: the DNA polymerase (22, 39), the helicase-primase (12), and the origin-binding protein (2, 15). It also appears to interact directly with the HSV-1 DNA polymerase-UL42 complex (8, 26, 34), the origin-binding protein (2), and the HSV-1 alkaline nuclease (41–43).

Another possible function for ICP8 is as a component of a recombination system. It is known that high levels of homologous recombination occur in HSV-1-infected cells (37), and inversion of the HSV-1 genome through internally repeated sequences also occurs during infection (14, 21, 28). Several single-strand binding proteins (SSBs) have been shown to participate in recombination, notably the *Escherichia coli* SSB and the T4 gene 32 protein (11). To investigate the possible role of ICP8 in recombination, we chose to examine renaturation of complementary single strands of DNA, one of the simplest of the DNA-pairing reactions associated with general

recombination. DNA renaturation has been shown to be promoted by the *E. coli* SSB (9) and the T4 gene 32 protein (1), as well as by such recombinases as the *E. coli* RecA (6, 27, 45) and RecT proteins (20), the phage λ β protein (24, 29), and the yeast strand exchange proteins, SEP1 and SF1 (23, 30). In this report, we demonstrate that ICP8 can also promote the renaturation of complementary single strands of DNA.

MATERIALS AND METHODS

Materials. ICP8 was purified from either HSV-1-infected U35 cells or SF9 cells infected with a recombinant baculovirus containing the ICP8 gene (40). Purification was performed as described by Hernandez and Lehman (22) for ICP8 from HSV-1-infected U35 cells or as described by Boehmer and Lehman (4) for ICP8 from recombinant baculovirus. In both cases, the protein was >95% pure, as judged by densitometry on Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels. Buffer A contained 1 mM dithiothreitol, 50 μ g of bovine serum albumin per ml, 5 mM $MgCl_2$, 5% glycerol, 75 mM NaCl, and 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.6.

DNA substrates. The pUC19 plasmid was prepared by the alkaline lysis procedure followed by purification on Qiagen columns according to the manufacturer's instructions. The DNA was then linearized with either *Eco*R1 or *Bam*HI (New England Biolabs), purified on a 1% agarose gel, and eluted from the gel with an Elutrap electrophoresis chamber (Schleicher and Schuell). Linearized pUC19 was labeled with [γ - 32 P]dCTP (3,000 Ci/mmol; New England Nuclear) by using the Sequenase version 2.0 protocol from U.S. Biochemical Corp. Unincorporated nucleotide was removed by spin column chromatography (Quickspin DNA, G-25; Boehringer). DNA was denatured by boiling for 5 min followed by rapid chilling on ice.

Renaturation assay. Unless otherwise indicated, 10- μ l reactions were performed at 37°C for 15 min in buffer A containing 0.43 nM DNA molecules and 0.06 μ M ICP8. Reaction components were mixed on ice, and the reaction was initiated by transfer to 37°C. Reactions were terminated by addition of 2.5 μ l of 0.5% sodium dodecyl sulfate–10% glycerol with added bromophenol blue, and the products were electrophoresed on a 1% Tris-acetate-EDTA agarose gel. Following electrophore-

* Corresponding author.

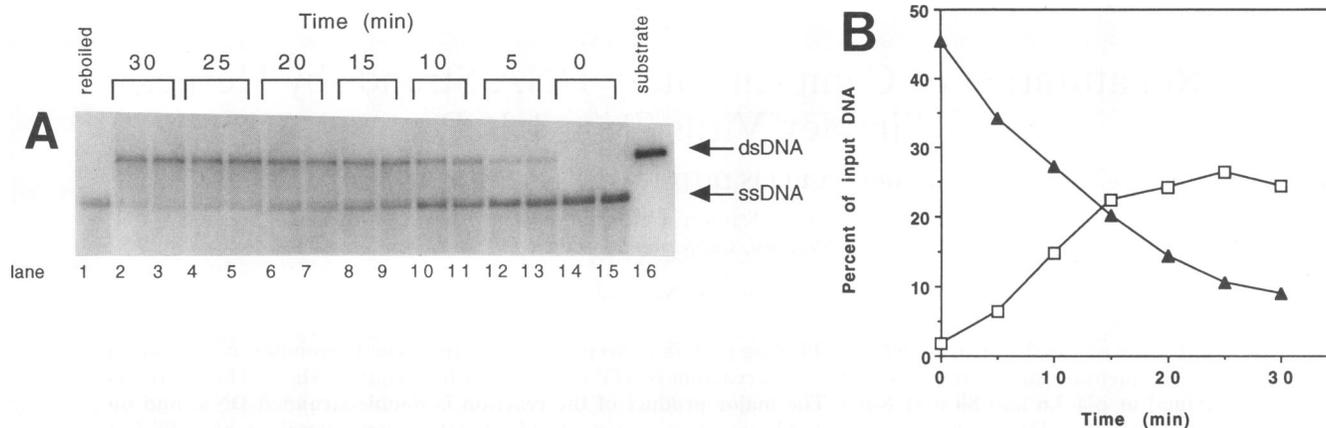


FIG. 1. Time course of ICP8-promoted renaturation reaction. Reactions were performed as described in Materials and Methods, except that 2 mM MgCl₂ and 50 mM NaCl were used. All points on the graph represent values for duplicate samples. (A) Autoradiographic analysis. Times of incubation with ICP8 are given above the lanes; for lane 1, the sample was reboiled prior to loading on the gel, and lane 16 contains the substrate prior to initial boiling. (B) Phosphoimage analysis. Percent total ssDNA (▲) or dsDNA (□) was calculated as described in Materials and Methods.

sis, the gels were dried on DE81 paper (Whatman) and quantitated with a phosphoimager (Molecular Dynamics). Percent total input DNA was calculated by dividing the number of counts in the ssDNA band or the double-stranded

DNA (dsDNA) band by the total number of counts used in the reaction. Percent dsDNA was calculated by dividing the number of counts in the dsDNA band by the total number of counts in the dsDNA and ssDNA bands.

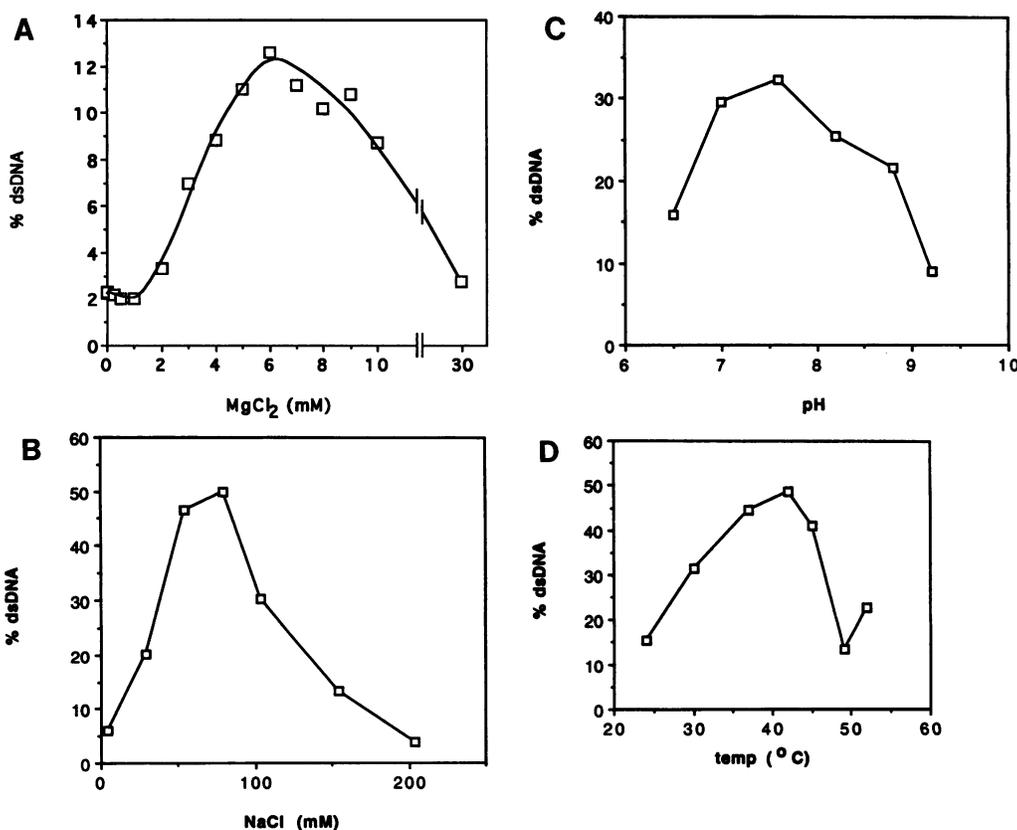


FIG. 2. Effects of MgCl₂, NaCl, pH, and temperature on renaturation reaction. All datum points represent values for duplicate samples. Percent dsDNA was calculated as described in Materials and Methods. (A) MgCl₂ titration. Reactions were performed as described in Materials and Methods, except that 50 mM NaCl was used. (B) NaCl titration. (C) pH titration. The following buffers were used at 40 mM: MES [2-(*N*-morpholino)ethanesulfonic acid], pH 6.5; MOPS [3-(*N*-morpholino)propanesulfonic acid], pH 7.0; HEPES, pH 7.6; EPPS [*N*-(2-hydroxyethyl)piperazine-*N'*-3-propane-sulfonic acid], pHs 7.6, 8.2, and 8.8; and TAPS [*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid], pH 9.2. (D) Temperature curve.

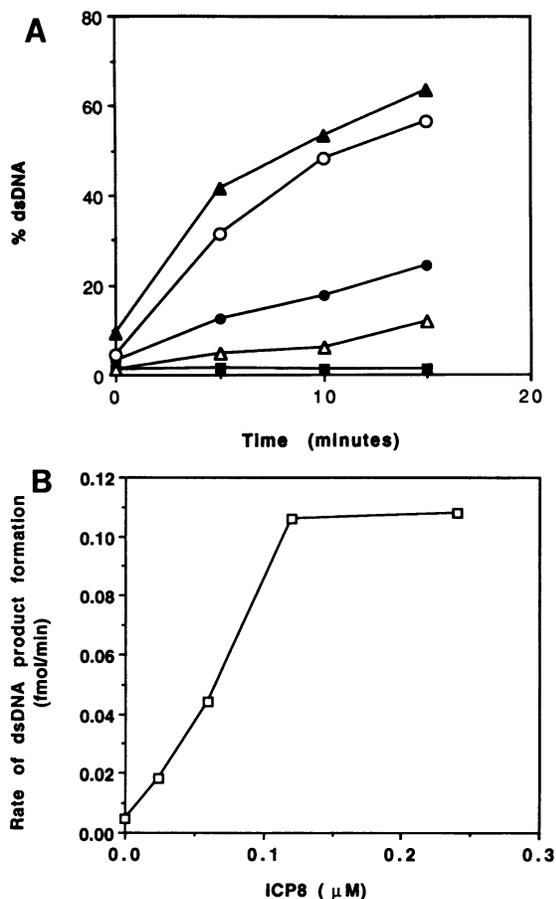


FIG. 3. Protein titration of ICP8-promoted renaturation reaction. Assays were performed as described in Materials and Methods. All points represent values for duplicate samples. (A) Time course of renaturation. Symbols: ■, no ICP8; △, 0.024 μM ICP8; ●, 0.058 μM ICP8; ○, 0.117 μM ICP8; ▲, 0.234 μM ICP8. (B) Rate of reaction during first 5 min at different ICP8 concentrations.

RESULTS

ICP8 promotes renaturation of complementary single strands. Linearized, end-labeled pUC19 dsDNA was heat denatured to produce ssDNA (Fig. 1A, lanes 14 and 15). Incubation of ICP8 with the ssDNA resulted in the gradual reappearance of a band corresponding in size to the double-stranded form of the DNA (Fig. 1A, lanes 2 to 13) and the concomitant disappearance of the single-stranded form. Subsequent boiling of the reaction mixture after a 30-min incubation with ICP8 resulted in loss of the dsDNA product and reappearance of the ssDNA substrate (Fig. 1A, lane 1), further confirming the identities of the two bands. Quantitation of the ssDNA substrate and the dsDNA product by phosphorimager analysis was then performed. As shown in Fig. 1B, 45% of the input DNA was present as ssDNA substrate at the start of the time course. Some smearing was seen below the ssDNA band, indicating that some of the initial pUC19 contained nicks. The percentage of DNA present as ssDNA substrate decreased throughout the time course, while the percentage present as dsDNA product increased rapidly during the first 15 min and then leveled off. Little DNA was detected in the wells, indicating that ICP8 renaturation of DNA does not result in the

large networks of DNA that are formed by the majority of strand-annealing proteins (20, 23, 30, 45).

Effects of Mg^{2+} , NaCl, pH, and temperature on the renaturation reaction promoted by ICP8. The renaturation reaction promoted by ICP8 requires Mg^{2+} , with little renaturation seen in its absence or at 0.25 mM MgCl_2 (Fig. 2A). Maximal renaturation activity occurred at 6 mM MgCl_2 , with higher concentrations resulting in inhibition.

Renaturation was optimal at 80 mM NaCl (Fig. 2B), with little activity at low NaCl concentrations and inhibition at concentrations above 100 mM. Renaturation occurred over a broad pH range, with an optimum level at pH 7.6 (Fig. 2C).

Little renaturation was seen at 0°C (data not shown), and analysis of the renaturation reaction at different temperatures demonstrated that maximal activity occurred at 40°C (Fig. 2D). However, further experiments were performed at 37°C, which produced nearly optimal renaturation and is the temperature at which ICP8 would be expected to act in vivo. ICP8 promoted the renaturation of complementary single strands varying in length from 2,686 (pUC19) to 476 bp, the smallest size tested (data not shown). ATP had no effect on the ability of ICP8 to promote renaturation (data not shown).

Effect of ICP8 concentration on renaturation. The time course of the renaturation reaction was examined at five different ICP8 concentrations. No renaturation of complementary single strands was observed in the absence of ICP8 during the 15-min incubation period (Fig. 3A). Addition of ICP8 stimulated renaturation even at the lowest concentration of ICP8 tested. An approximately linear increase in product formation was observed up to 0.12 μM ICP8, an amount corresponding to one ICP8 per 20 DNA nucleotides. This value agrees with the stoichiometry of one ICP8 per 22 nucleotides of ssDNA determined previously (22). Thus, the renaturation activity of ICP8 appears to be maximal at saturating levels of the protein. ICP8 concentrations up to 0.7 μM gave the same amount of renaturation as 0.12 μM ICP8 (data not shown), indicating that greater-than-saturating levels of the protein are not inhibitory.

Effect of varying DNA concentration on renaturation. To determine the effect of varying DNA concentration on ICP8-promoted renaturation, the time course of the reaction was

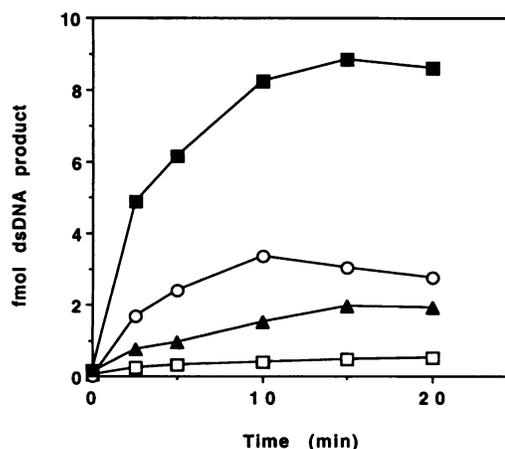


FIG. 4. Effect of DNA concentration on renaturation reaction. Assays were performed as described in Materials and Methods. All points represent values for duplicate samples. 0.7 μM ICP8 was added to each sample. Symbols: □, 0.29 nM pUC19; ▲, 0.58 nM pUC19; ○, 0.82 nM pUC19; ■, 1.45 nM pUC19.

examined at four different DNA concentrations. The ICP8 in the reaction mixture was kept constant at 0.7 μ M, an amount sufficient to ensure that the protein was present in excess. As shown in Fig. 4, the amount of dsDNA product formed was dependent on the concentration of DNA. The rate of dsDNA formation during the first 2.5 min was 0.082 fmol/min for the 0.29 nM DNA samples (the concentration is that of the pUC19 molecules), 0.253 fmol/min for the 0.58 nM DNA samples, 0.676 fmol/min for the 0.82 nM DNA samples, and 1.88 fmol/min for the 1.45 nM DNA samples. Thus, the rate of product formation was proportional to the square of the DNA concentration, indicating that the renaturation reaction promoted by ICP8 is second order relative to DNA concentration.

DISCUSSION

ICP8, the major ssDNA-binding protein of HSV-1, can promote renaturation of complementary single strands of DNA. The reaction does not require ATP but is dependent on Mg^{2+} . Optimal conditions include 80 mM NaCl and a pH of 7.6. The major product appears to consist of two complementary strands annealed to form dsDNA; no large DNA networks are detectable in the wells. Maximal renaturation is reached with saturating levels of ICP8, and no inhibition is seen with ICP8 added above saturation. The reaction is second order with respect to DNA concentration.

Several other DNA-binding proteins can promote the renaturation of complementary single strands. Like ICP8, the *E. coli* SSB and the phage T4 gene 32 protein promote renaturation in the absence of ATP and give optimal renaturation when the protein is present at saturating levels (1, 9). ICP8 also resembles the *E. coli* SSB and the phage T4 gene 32 protein in that the renaturation reaction is second order relative to DNA concentration (1, 9), similar to the renaturation of DNA in the absence of added protein (47). In contrast, the *E. coli* RecA protein is stimulated by ATP at moderate Mg^{2+} levels, promotes renaturation most efficiently at subsaturating levels of protein (6, 45), and shows first-order kinetics. The second-order kinetics observed with ICP8 indicate that the rate-limiting step in the reaction is the nucleation event (initial formation of a few correct base pairs between the two strands) (47). ICP8 could promote renaturation by melting out intramolecular secondary structure, by its known ability to hold the DNA in extended conformation (38), or by direct protein-protein or protein-DNA interactions which facilitate DNA base pairing. Further studies are clearly needed to determine the exact mechanism of ICP8-promoted renaturation. The dsDNA nature of the product of ICP8-promoted renaturation is seen only with one other ssDNA-binding protein, the heterogeneous nuclear ribonucleoprotein A1 (33). All other proteins which promote renaturation yield large aggregates of DNA as their final products. The reason for this difference is unknown.

ICP8 can unwind short DNA duplexes (3); however, the conditions for maximal unwinding (no $MgCl_2$ or NaCl) give almost undetectable renaturation, and little unwinding is seen under conditions optimal for renaturation (6 mM $MgCl_2$, 80 mM NaCl). Very recently, ICP8 has been shown to catalyze strand exchange (5). The renaturation and strand exchange activities of ICP8 suggest that ICP8 may play a role in the high level of recombination that occurs during HSV-1 infection.

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