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Tumorigenic transformation induced by a specific fragment of DNA from herpes simplex virus type 2*

(viral oncogenesis/restriction endonucleases/oncogenic fragment/antigen AG-4)

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ABSTRACT **Transfection of Syrian hamster embryo cells with limit digests of *Bgl* II-, *Hpa* I-, or *Bgl* II/*Hpa* I-cleaved DNA from herpes simplex virus type 2 (strain S-1) but not with salmon sperm DNA resulted in the appearance of refractile, morphologically altered cells at a frequency of 10^{-5}/0.005 μg of viral DNA within two to four passages. Transformed lines manifested reduced serum requirement and anchorage-independent growth and were tumorigenic in newborn hamsters. They expressed ICP10, a viral protein immunologically identical to the cervical-tumor-associated AG-4 antigen. Transforming activity was localized in the 16.5 × 10^6-dalton *Bgl* II/*Hpa* I double-digest fragment CD_{S-1}, which exhibited sequence homology to the *Bgl* II/*Hpa* I fragment CD of DNA from herpes simplex virus type 2 strain 333, mapping between coordinates 0.43 and 0.58 on the physical map of strain 333 DNA. This fragment, CD_{333}, was also shown to induce neoplastic transformation.**

Inactivated herpes simplex virus type 2 (HSV-2) (1, 2) and low concentrations of native, noninactivated viral DNA (3, 4) induce the neoplastic transformation of mammalian cells in culture. Transformation appears to be mediated by fragment(s) of the HSV-2 genome: (*i*) mechanically sheared HSV-2 DNA (M_r 9 × 10^6) induces neoplastic transformation at a frequency identical to that observed with native DNA (4), (*ii*) hamster cells transformed by UV-irradiated HSV-2 retain a fraction (8–32%) of the viral DNA sequences (5), and (*iii*) virus transforming activity is more resistant than its infectivity to radiation damage (1, 2). However, the oncogenic fragment in the HSV-2 genome has not yet been identified.

Provided that oncogenic activity does not depend on distant noncontiguous genes and that suitable restriction endonucleases can yield appropriate fragments of HSV-2 DNA that do not express lytic functions, it seems reasonable to propose that neoplastic transformation should be demonstrable with a restriction endonuclease fragment. The data described in this report support the validity of this proposal.

MATERIALS AND METHODS

Cells and Virus. Syrian hamster embryo (SHE) cells were grown in ERM medium with 10% fetal bovine serum (3). Vero and HEp-2 cells were grown in medium 199 with 10% fetal bovine serum. HSV-2 strain S-1, a human cervical tumor isolate (6), was plaque-purified and grown in HEp-2 cells at a low (0.1 plaque-forming unit/cell) multiplicity of infection. HSV-2 strain 333 was obtained from R. Duff.

DNA Purification. Viral DNA was purified from Vero cells infected with HSV-2 strains S-1 or 333 by buoyant density centrifugation in NaI gradients containing ethidium bromide as described (3, 7).

DNA Restriction and Fractionation. Viral DNA (10 μg) was incubated with 30–50 units of *Bgl* II (New England BioLabs, Beverly, MA), or *Hpa* I (Bethesda Research, Rockville, MD) in 10 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/6 mM KCl/1 mM dithiothreitol. Reaction was terminated by adding 0.2 vol of 0.1 M EDTA/50% sucrose/0.1% bromophenol blue and the fragments were separated by electrophoresis on cylindrical (20 × 1 cm) or horizontal slab (30 × 13 × 0.6 cm) 0.3% agarose gels in Tris/phosphate buffer containing 0.5 μg of ethidium bromide per ml as described (8). A *Hin*dIII digest of λ DNA (New England BioLabs), simultaneously electrophoresed, was used as a molecular weight marker.

To isolate fragments, we crushed gel slices in buffer (10 mM Tris·HCl/1 mM EDTA/0.1% $NaDodSO_4$, pH 8.0) and incubated them overnight at 45°C. Agarose was removed by centrifugation at 10,000 rpm for 20 min, and the supernatant was extracted with phenol/chloroform/isoamyl alcohol and dialyzed against Hepes-buffered saline, pH 7.05 (9). DNA concentration was determined by UV absorbance at 260 nm. Recovery was approximately 75%.

Transformation. Cells were transfected with restriction-endonuclease-cleaved HSV-2 (S-1) DNA deproteinized by phenol extraction or with specific HSV-2 (S-1) DNA fragments and assayed for transformation-associated properties as described (3, 4).

***In Vitro* Labeling of HSV-2 DNA Fragment.** One 16.5 × 10^6-dalton fragment designated CD_{S-1} (Fig. 2, gel 3), obtained by *Bgl* II/*Hpa* I double digestion of HSV-2 (S-1) DNA, was labeled *in vitro* by a modification of the nick translation procedure (10). Reaction mixtures (100 μl) contained 1–2 μg of purified fragment CD_{S-1}, 5 μmol of sodium phosphate (pH 7.6), 1 μmol of $MgCl_2$, 1 μmol of 2-mercaptoethanol, 0.1 μmol of EDTA, 20 μg of bovine serum albumin, 200 μCi each of [α-^{32}P]dCTP and [α-^{32}P]dGTP (100–200 Ci/mmol, ICN Chemical and Radioisotope; 1 Ci = 3.7 × 10^{10} becquerels) 10 μmol each of unlabeled dATP and dTTP, and 15 units of *Escherichia coli* DNA polymerase I (Boehringer grade I, 2500–5000 units/mg). They were incubated at 14°C until 20% of radioactivity was incorporated. Unincorporated triphosphates were removed on Sephadex G-100.

Hybridization. ^{32}P-Labeled CD_{S-1} fragment was hybridized as described by Pellicer *et al.* (11) to unlabeled *Bgl* II/*Hpa* I fragments of HSV-2 (S-1) and HSV-2 (333) DNA that had been transferred from 0.3% agarose gels to nitrocellulose filters by the procedure of Southern (12).

Antigens and Antisera. Antisera to ICP10 or total viral antigens (Ra-2) were prepared in rabbits by subcutaneous inoculation in the presence of complete Freund's adjuvant (13).

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Abbreviations: HSV-2, herpes simplex virus type 2; SHE cells, Syrian hamster embryo cells.

* A preliminary report of these results was presented at the Fourth Meeting on Herpesviruses, Cold Spring Harbor, NY, August 1979.

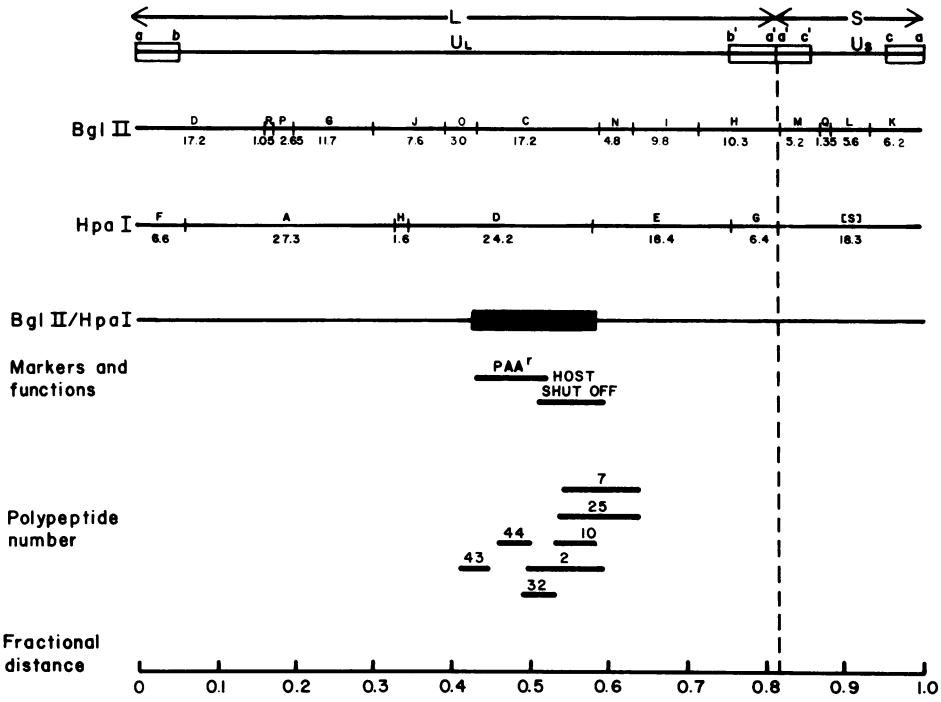

FIG. 1. Location of *Bgl* II and *Hpa* I fragments in the prototype arrangement of HSV-2 333 DNA based on published maps for these sites (8, 17). *Bgl* II digestion products of our 333 DNA were identical to those expected from the published maps (Fig. 2). The identity of individual *Bgl* II digestion products was confirmed by redigestion of DNA with *Hpa* I. The solid block indicates location of the transforming fragment obtained by *Bgl* II/*Hpa* I double digestion. Sequence homology is observed between this fragment from S-1 and 333 DNAs (Fig. 3).

ICP10 is an "immediate early" viral protein (14) purified to radiochemical homogeneity by $NaDodSO_4$/acrylamide gel electrophoresis and shown to be immunologically identical (13) to AG-4, the virus-specific antigen that reflects the progression of human squamous cervical cancer (15). Prior to use in these studies, Ra-2 serum was repeatedly adsorbed with uninfected HEp-2 cells (15), and all antisera were fractionated on linear 10–40% (wt/vol) sucrose gradients as described (13). The IgG fractions were used in all assays.

Complement Fixation Assay. The high sensitivity and reproducibility of the microquantitative complement fixation assay have been described (16). The assay was performed in triplicate with optimal concentrations of antigen (20 μg of protein) and IgG (1.4 dilution) (16). Reaction was considered positive if more than 10% of the complement was fixed (13, 16).

RESULTS

Restriction Endonuclease Digestion of HSV-2 DNA. *Bgl* II and *Hpa* I restriction sites in the DNA of the G (8, 17) and 333 (Third Herpesvirus Workshop, Cold Spring Harbor, NY, September, 1976) strains of HSV-2 and the resulting cleavage maps (Fig. 1) are known (8, 17). The *Bgl* II digestion products of DNA from our strain 333 (333 DNA), analyzed on 0.3% agarose, were identical to those expected from the published maps (Fig. 2). DNA of strain S-1 digested by *Bgl* II yielded a similar cleavage pattern except that it lacked a band corresponding to fragment G of 333 DNA and displayed a decrease in the relative mobility of the band corresponding to fragment L of 333 DNA. However, there are three new bands in size class V of S-1 DNA (Fig. 2) that together have a sum of relative M_r of 10.7×10^6, which approximates the M_r (11.7×10^6) of the absent G fragment. This observation is consistent with reported (17, 18) differences among the digestion patterns of DNAs from various HSV strains that represent acquisition or loss of a cleavage site and fluctuations in the relative mobility of certain fragments. Although the exact identity of the new bands in S-1 DNA is unknown, additional cleavage sites in the G fragment might account for the three new bands in size class V of S-1 DNA. For the sake of the present discussion, we shall assume that fragments from the *Bgl* II digests of S-1 and 333 DNA having identical relative mobility in 0.3% agarose gels are equivalent. Sequence homology between the transforming region of S-1 DNA and the corresponding 333 DNA fragment is demonstrated by hy-

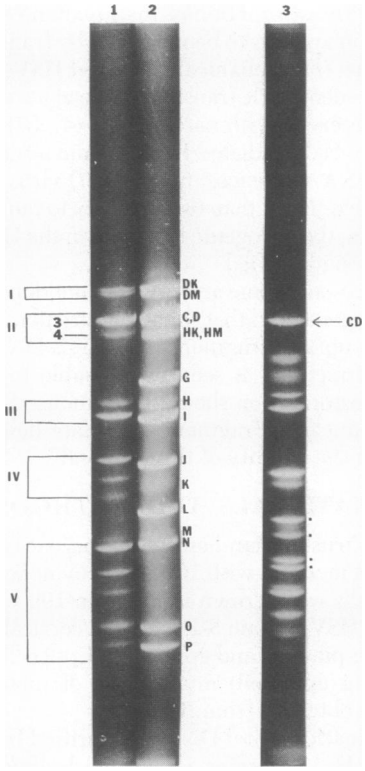

FIG. 2. Electrophoretic separation of HSV-2 DNA from strains S-1 and 333 digested with *Bgl* II and *Bgl* II/*Hpa* I and stained with ethidium bromide. Roman numerals and numbers refer to fragment size classes and bands, respectively, in the *Bgl* II digest of S-1 DNA. Lane 1, *Bgl* II digestion products of S-1 DNA (2.8 μg); lane 2, *Bgl* II digestion products of 333 DNA (5.7 μg); lane 3, *Bgl* II/*Hpa* I digestion products of S-1 DNA (5.8 μg). Black dots represent fragments of relative mobility different from that of 333 DNA.

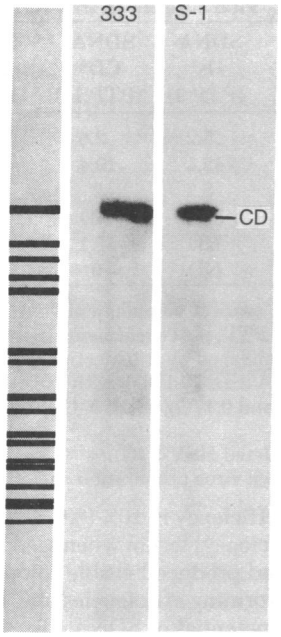

FIG. 3. Nitrocellulose filter hybridization of S-1 and 333 DNAs after restriction endonuclease digestion and denaturation *in situ*. *Bgl* II/*Hpa* I double digests of S-1 (5.3 μg) and 333 (9 μg) DNA were electrophoresed on 0.3% agarose gels, denatured *in situ*, and transferred to nitrocellulose filters (12). These were soaked in Denhardt's buffer containing 10 μg of heat-denatured *E. coli* DNA per ml and annealed with ^{32}P-labeled *Bgl* II/*Hpa* I CD_{S-1} fragment as described (11). (*Left*) Guide strip showing fragments of *Bgl* II/*Hpa* I-digested S-1 DNA as resolved on a 0.3% agarose gel. (*Middle*) Hybridization reaction between unlabeled *Bgl* II/*Hpa* I fragments of 333 DNA and ^{32}P-labeled CD_{S-1} fragment. (*Right*) Hybridization reaction between unlabeled *Bgl* II/*Hpa* I fragments of S-1 DNA and ^{32}P-labeled fragment CD_{S-1}.

bridization (Fig. 3). For the purpose of identification, a subscript "S-1" will be added to the letter designating a specific fragment of S-1 DNA, such as C_{S-1}, D_{S-1}, etc.

Transformation by Total Digests. The transforming activity of unfractionated limit digests of restriction-endonuclease-cleaved HSV-2 (S-1) DNA was assayed at concentrations similar to those that we previously used for native and mechanically sheared HSV-2 (S-1) DNA (3, 4). Unfractionated digests of *Bgl* II-cleaved HSV-2 (S-1) DNA (0.01–1.0 μg) were precipitated with $CaCl_2$ and applied to SHE cells. Within two to four passages after transfection, refractile, morphologically altered (predominantly stellate) cells were detected in the transfected but not in the mock-infected (medium or salmon sperm DNA) cultures (Table 1). They overgrew the cultures within two to six further passages. Under identical conditions, transformation was also observed with 0.1 and 0.01 μg of unfractionated *Hpa* I limit digests and with 0.001 and 1.0 μg of total double digests of *Bgl* II/*Hpa* I-cleaved HSV-2 (S-1) DNA. Because the unique morphological features of HSV-transformed colonies have not been rigorously defined, escape from senescence was used as a more reliable criterion for transformation. Assuming that at least one out of 5×10^5 seeded cells per 60-mm dish was transformed in a culture exposed to 0.001 μg of *Bgl* II/*Hpa* I digest, it may be estimated that the frequency of transformation is $1/10^5$ cells per 0.005 μg of DNA (Table 1).

Table 1. Transforming activity of total restriction digests of HSV-2 (S-1) DNA

Unfractionated digest	μg DNA per dish*	No. transformed cultures/total no. cultures
Bgl II	1.0†	4/4
	0.1	2/2
	0.01	2/2
Hpa I	0.1†	4/4
	0.01†	4/4
Bgl II/*Hpa* I	0.1	2/2
	0.001	2/2
Untreated or control‡		0/12§

* Sufficient salmon sperm DNA was added to bring final DNA concentration to 5 μg per 60-mm dish.
† Results from two independent experiments.
‡ Received 5 μg of salmon sperm DNA or medium only.
§ In HSV transformation experiments thus far, no spontaneously transformed cell line has been established from more than 30 untreated or control cultures.

Table 2. Transforming activity of restriction enzyme fragments of HSV-2 (S-1) DNA

Bgl II size class or *Bgl* II/*Hpa* I fragment	μg DNA/dish			No. transformed cultures/total no. cultures		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
I	0.01	0.04	0.46	0/4	0/2	0/2
II (bands 3 and 4)	0.026			2/2		
II (band 3)	0.20	0.10	1.14	4/4	2/2	2/2
	0.02	0.02	0.006	4/4	2/2	2/2
			0.002			2/2
II (band 4)		0.03	0.34		0/2	0/2
		0.005			0/2	
III	0.01	0.06	0.47	0/4	0/2	0/2
IV	0.01	0.06	0.47	0/4	0/2	0/2
V	0.01	0.10	1.14	0/4	0/2	0/2
CD_{S-1} fragment		0.18	0.124		2/2	2/2
			0.012			4/4

Transformation by Fragments. HSV-2 (S-1) DNA cleaved with *Bgl* II was electrophoresed on 0.3% agarose gels and the fragments were divided into five size classes (Fig. 2). These were eluted and used to transfect SHE cells. Within the concentration range (0.005–1.14 μg) studied in this series, SHE cell transformation was observed only with *Bgl* II fragments in size class II. *Bgl* II fragments in size classes I, III, and V did not transform even at concentrations 18- to 44-fold higher than that (0.026 μg) at which size class II was active (Table 2).

Bgl II fragments in size class II consist of two bands designated 3 and 4 (Fig. 2). These were independently eluted and assayed for transforming activity. Transformation was observed only in cultures transfected with 0.002–1.14 μg of size class II band 3 DNA (Table 2), which, by analogy to 333 DNA, is expected to consist of two fragments, C_{S-1} and D_{S-1} (17.2×10^6 daltons). Transformation was not detected in cells transfected with 0.005–0.34 μg of band 4 DNA, which, by analogy to 333 DNA, is expected to consist of two fragments, HK_{S-1} and HM_{S-1} (16.5×10^6 and 15.5×10^6 daltons, respectively) (Figs. 1 and 2).

Localization of the transforming activity within one of the two 17.2×10^6-dalton fragments (C_{S-1} and D_{S-1}) in band 3 of S-1 DNA depends on their separation according to criteria other than molecular weight. Because the sequence of fragment D_{S-1} is also present in nontransforming size class I as DK_{S-1} and DM_{S-1} fragments (Fig. 2), it seems reasonable to conclude that the transforming potential of band 3 is associated only with the C_{S-1} fragment. Accordingly, further digestion of the *Bgl* II-cleaved DNA by an enzyme that cleaves within D_{S-1} but not C_{S-1} should result in the purification of the latter fragment

Table 3. Phenotypic characterization (3) of transformed hamster cell lines

	Normal SHE (P 3–5)	SDNA-1B (PTP 15–25)	SDNA-CD (PTP 13–48)
Morphology	Fibroblasts	Predominantly stellate	Stellate
Cloning efficiency,* %			
In 10% serum	0	32.4	26
In 2% serum	0	19.8	26
Colony-forming efficiency, %			
In 0.3% agarose	0	10.2	24
In 0.3% agar	0	4.8	22.8
Tumorigenicity†	0	100 (7/7)	100‡ (4/4)

P, passage; PTP, post-treatment passage.
* At low plating density (100–500 cells per 60-mm dish).
† Neonatal hamsters were inoculated subcutaneously with 2×10^6 viable cells and palpated twice weekly for 180 days for tumor development.
‡ Tumor incidence at PTP 48.

without affecting the transforming potential of the DNA. *Hpa* I fulfills these requirements: it cleaves D_{S-1} into fragments of 10.6×10^6 and 6.6×10^6 daltons while reducing C_{S-1} by only 5% (i.e., to a 16.5×10^6-dalton fragment designated CD_{S-1}, or fragment A, old designation) (Fig. 1). Furthermore, *Hpa* I cleaves within the H fragments (Fig. 1) generated by *Bgl* II (such as HK_{S-1}, and HM_{S-1}, 16.5×10^6 and 15.5×10^6 daltons, respectively), thus eliminating any possible contamination of the 16.5×10^6-dalton CD_{S-1} fragment generated by double digestion with *Bgl* II/*Hpa* I.

The general cleavage patterns of *Bgl* II/*Hpa* I double digests of 333 and S-1 DNA were similar. Minor differences were observed only in the relative mobility of four bands located within a M_r range of $4.2–7.5 \times 10^6$, a range lower than that expected for fragment CD_{S-1}, which has a M_r of 16.5×10^6 (Fig. 2). The identity of the CD fragment of S-1 DNA to that of 333 DNA, both generated by *Bgl* II/*Hpa* I double digestion, was confirmed by Southern blot hybridization (12). Nitrocellulose blots of *Bgl* II/*Hpa* I fragments of S-1 and 333 DNA were annealed to ^{32}P-labeled CD fragment prepared from S-1 DNA. The fragment hybridized with only one band of 333 or S-1 DNA in the gel; this band displayed a relative mobility identical to that of *Bgl* II/*Hpa* I-generated CD_{S-1} fragment (Fig. 3). Within two to four passages SHE cells transfected with 0.012–0.18 μg of the *Bgl* II/*Hpa* I CD_{S-1} fragment (Table 2) displayed morphologically altered (stellate and more refractile) cells which overgrew the cultures and produced rapidly growing lines within six to eight further passages.

The 16.5×10^6-dalton fragment CD_{333} obtained by *Bgl* II/*Hpa* I double digestion of the DNA of the 333 strain was assayed for transforming activity. Morphological alteration was detected within two passages in all four cultures transfected with 0.007–0.02 μg of CD_{333} fragment but not in those exposed to 5 μg of salmon sperm DNA. One rapidly growing cell line was established from a culture transfected with 0.007 μg of CD_{333} DNA fragment.

Phenotypic Properties of Cells Transformed by Fragmented HSV-2 DNA. Transformed lines established with unfractionated digests of *Bgl* II-cleaved HSV-2 (S-1) DNA (SDNA-1B and SDNA-0.1B) or with CD_{S-1} (SDNA-CD) or CD_{333} (333 DNA-CD) fragments generated by double digestion with *Bgl* II/*Hpa* I were examined for phenotypic alterations associated with the neoplastic state (Table 3) and for the presence of viral antigens (Table 4). Transformed lines exhibited

Table 4. Viral antigens in transformed cells

IgG	SHE	SDNA-1B* (PTP 9)	SDNA-CD* (PTP 13)	SDNA-CD1* (PTP 39)	SDNA-CD2* (PTP 14)
Preimmune	0	5.2	2.6	7.6	1.3
Anti-ICP10	0	42.4	19.4	25.0	29.2
Anti-ICP10†	5.9	8.2	2	ND	ND
Anti-ICP10‡	ND	35.4	ND	ND	ND
Ra-2	0	ND	55.1	49.2	61.5
Preimmune	7	ND	0	0	1.2

Values shown are percent complement fixed by the various cell lines. ND, not done. PTP, post-treatment passage.
* Cell lines were established by transfection with 1.0 μg of total *Bgl* II digest of S-1 DNA (SDNA-1B) or with 0.012 μg (SDNA-CD), 0.12 μg (SDNA-CD1), and 0.17 μg (SDNA-CD2) of *Bgl* II/*Hpa* I fragment CD_{S-1}.
† Adsorbed with pelleted HSV-2 (G).
‡ Adsorbed with mock virus preparation.

increased cloning efficiency in 10% (8.8–43.5% efficiency) and 2% (2.8–19.8% efficiency) serum when seeded at 100–500 cells per 60-mm dish and produced visible colonies in agarose and agar with colony-forming efficiencies of 0.16–24%.

The oncogenic potential of SDNA-1B, 333 DNA-CD, and SDNA-CD cells was investigated by inoculating 2×10^6 cells subcutaneously into 1- to 3-day old Syrian hamsters. Tumors (fibrosarcomas and tumors of epithelial origin) were detected at the site of inoculation within 5 weeks in 43% of the animals inoculated with SDNA-1B cells at post-treatment passage 25. By 9 weeks, all the inoculated animals (7/7) were positive for tumors. Fibrosarcomas (1.5–2.5 cm) showing signs of local invasion into the clavicular muscle region were also detected within 5 weeks in 66% of the animals inoculated with 333 DNA-CD cells at post-treatment passage 39. SDNA-CD cells inoculated at post-treatment passage 48 induced nonregressive invasive fibrosarcomas in 100% of the animals within a latent period of 5½ weeks.

Extracts from SDNA-1B and SDNA-CD cells fixed complement with IgG from Ra-2 and anti-ICP10 sera. The reaction was virus specific, as shown by the observations that preimmune rabbit IgG was nonreactive and that anti-ICP10 and Ra-2 IgG did not fix complement with SHE cell extracts. Anti-ICP10 IgG fixed complement with pelleted HSV-2 (G) but not with mock-virus preparations (15), and its reactivity was specifically adsorbed by the former but not the latter antigen (Table 4). SDNA-CD1 cells remained positive for viral antigen expression at post-treatment passage 39 (approximately 120–150 population doublings).

DISCUSSION

These studies demonstrate that SHE cells are neoplastically transformed by a specific restriction endonuclease fragment of the DNA of HSV-2 (S-1), a virus originally isolated from intraepithelial cervical carcinoma (6). The transforming potential, the identity, and the expression of this fragment in the transformed cells merit discussion.

DNA fragments were studied at concentrations (0.005–1.14 μg/dish) previously used in transformation by native (3) or randomly fragmented (4) S-1 DNA. Transforming activity was displayed only by band 3 in size class II of *Bgl* II-cleaved S-1 DNA (Table 2). Because all DNA fragments were used within the same concentration range (0.01–0.46 μg/dish), the failure to induce transformation with DNA fragments other than band 3 (Table 2) is probably due to differences in their transforming potential. Thus, band 4 in size class II failed to yield transformation even at a concentration (0.34 μg/dish) 170-fold higher than that (0.002 μg/dish) at which band 3 was still active.

Therefore, band 3 is at least 170-fold higher in transforming potential (or "specific transforming activity") than band 4. Relative or absolute quantitative measurements of the transforming potential of all fragments are not yet feasible, being complicated by the very small amount of individual fragments obtainable within a size class or by the possible inhibitory effects of comigrating fragments within a size class. However, our data indicate that the transforming potential of other DNA fragments must be 1/20th–1/170th that of band 3 in size class II. Significantly, the specific transforming activity of band 3 DNA is comparable or higher than that of native (3) or randomly fragmented (4) S-1 DNA, suggesting that it may account for their transforming potential.

We have identified the oncogenic fragment as 16.5×10^6-dalton DNA generated by double digestion with *Bgl* II and *Hpa* I. Because the restriction map of S-1 DNA has not been fully characterized, determination of the fragment location within the viral genome depends on the determination of homology in sequence and transforming activity between the transforming fragment CD from S-1 DNA and the corresponding fragment from 333 DNA, the restriction map of which is known. Such knowledge allows the localization of the transforming fragment within the 333 DNA; its location in S-1 DNA would then depend on the colinearity between 333 and S-1 DNA.

Cleavage patterns of 333 DNA generated in our laboratory (Figs. 1 and 2) were identical to those published by other investigators (8, 17). By comparison, only minor differences (such as loss of the *Bgl* II G fragment) were observed in the cleavage patterns of S-1 DNA. The transforming CD fragment from S-1 DNA is uniquely homologous in sequence with the corresponding CD fragment from 333 DNA (Fig. 3) both generated by double *Bgl* II/*Hpa* I digestion. Furthermore, we have confirmed the neoplastic transforming activity of this CD_{333} fragment. Therefore, both from the standpoint of sequence homology and transforming activity, the transforming fragment CD_{333} is located within the map units 0.43–0.58 of 333 DNA. It remains to be determined whether other fragments from 333 DNA have transforming activity; in S-1 DNA, the transforming activity was found only in the $CD_{S\text{-}1}$ fragment.

The location of the transforming $CD_{S\text{-}1}$ fragment in S-1 DNA cannot be established with certainty. The minor differences in the cleavage patterns (Fig. 2) of DNA from strains 333 and S-1 [a biologically different virus strain (19)] are consistent with the reported variability in enzyme cleavage sites of DNA from different strains (17, 18). Establishment of colinearity between the sequences of S-1 and 333 DNA will involve hybridization of the S-1 DNA fragments with radioactive restriction fragments of 333 DNA. However, it should be stressed that presently published data support the colinearity concept. Thus, variation at each cleavage site occurs independently of other sites (17, 18), and interchange of DNA regions in intertypic (HSV-1 and HSV-2) recombination does not affect, at least grossly, the order of other genes (8, 17). Therefore, it is likely that the transforming $CD_{S\text{-}1}$ fragment is also located in the restriction map of S-1 DNA within coordinates 0.43–0.58.

The *Bgl* II/*Hpa* I fragment CD is sufficient in size to code for 10–15 proteins. Seven polypeptides of unknown functions, phosphonoacetic acid resistance, and host shut-off genes, unlikely candidates for transformation, have been mapped in this region (20). We find (Table 4) that at least ICP10 is expressed in SDNA-CD cells. This is a likely candidate for a transformation-specific protein because it reflects the progression of human cervical carcinoma (15) and it is an immediate early protein, as defined by its preferential synthesis in cells infected with HSV-2 in the presence of cycloheximide added at 0 hr and sequentially exposed to actinomycin D (14).

Camacho and Spear (21) reported recently that sequences in HSV type 1 DNA located between 0.3 and 0.45 map units were capable of inducing morphological transformation of hamster embryo cells. However, the neoplastic potential of these cells is unknown. Because there is only a small overlap between our region and that described by Camacho and Spear, it is probable that the HSV transforming gene(s) is located at different map positions in the two virus serotypes. Note that different aspects of transformation (i.e., morphologic in contrast to neoplastic) were investigated in these two studies.

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