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Multistep transformation by defined fragments of herpes simplex virus type 2 DNA: Oncogenic region and its gene product

(tumorigenic conversion/infected cell protein 10/ribonucleotide reductase/oncogene expression)

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ABSTRACT **Diploid Syrian hamster embryo cells transfected with *Bgl* II C fragment of herpes simplex virus type 2 DNA acquired a neoplastic phenotype. Cultures transfected with its left-hand 64% subclone *Eco*RI/*Hin*dIII fragment AE (0.419–0.525 map unit) grew into established but nontumorigenic lines. Transfection of *Eco*RI/*Hin*dIII AE-immortalized cells with a 4.4-kilobase *Sac* I/*Bam*HI subfragment within *Bam*HI E (0.554–0.584 map unit; overlaps the right-hand 16% of *Bgl* II C) converted them to tumorigenicity. The 4.4-kilobase subfragment encodes a 144-kDa protein immunologically and structurally similar to an infected cell protein designated ICP 10. DNA extracted from cells transformed with the 4.4-kilobase subfragment exhibited discrete hybridizing bands homologous to *Bam*HI E fragment. Monoclonal antibody to ICP 10 precipitated a 144-kDa protein from the transformed cells and stained them in immunofluorescence. A tumor derivative established with the transformed cells did not stain with this antibody, but ≈25% of the cells stained with a monoclonal antibody to c-*myc* protooncogene products.**

Transfection with isolated restriction endonuclease fragments has identified two distinct transforming regions (*Bgl* II fragments C and N, Fig. 1) in herpes simplex virus type 2 (HSV-2) DNA (1–4). The *Bgl* II N fragment [0.580–0.625 map unit (m.u.)] was reported to cause focus formation in rat embryo and NIH 3T3 cells (2, 4). The *Bgl* II C fragment (0.416–0.580 m.u.) was cloned in pBR322 as pGR140. It transforms normal diploid Syrian hamster embryo (SHE) cells in the continuous passage assay (1, 3) by a process that seemingly requires two steps (3). Thus, *Hin*dIII cleavage of the *Bgl* II C fragment (yielding two fragments, 0.416–0.525 and 0.525–0.580 m.u., Fig. 1) did not abrogate its tumorigenic potential. In addition, *Eco*RI/*Hin*dIII fragment AE (0.419–0.525 m.u.), cloned as pGR75 and representing the left-hand 64% of the *Bgl* II C fragment, induced escape from senescence ("immortalization"). However, the cells remained nontumorigenic, thereby raising obvious questions pertaining to the biological effect of the right-hand side of *Bgl* II C, which is now represented by the cloned *Bam*HI E fragment (pGH17a, 0.533–0.584 m.u., Fig. 1).

The present study was designed to (*i*) determine whether *Bgl* II C-induced neoplastic transformation of normal diploid SHE cells is a multistep process and (*ii*) identify the sequences that impart tumorigenic potential. The data indicate that a 4.4-kilobase (kb) subfragment located within the *Bam*HI E fragment of HSV-2 DNA (0.554–0.584 m.u.) and overlapping 16% of the right hand of the *Bgl* II C fragment converts cells immortalized by fragment *Eco*RI/*Hin*dIII AE to a fully tumorigenic phenotype. The 4.4-kb subfragment encodes a 144-kDa protein that is antigenically and structurally similar to a HSV-2-infected cell protein (ICP 10) that appears to be at least one of the components of the viral ribonucleotide reductase (5). A tumor derivative established with these transformed cells expresses the c-*myc* oncogene.

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MATERIALS AND METHODS

Cells. SHE cells, prepared from 13-day embryo, were obtained from T. Okeda and grown in IBR modified Dulbecco's Eagle reinforced medium with 10% fetal calf serum. HEp-2 cells were grown in medium 199 with 10% calf serum. K562 cells were grown in suspension as described (6).

Recombinant Plasmid DNA. Plasmids derived from pBR322 that contain fragments of HSV-2 (333) DNA in the 0.416–0.584 m.u. region (Fig. 1) were obtained from G. S. Hayward (The Johns Hopkins University, Baltimore, MD). The plasmids were propagated in *Escherichia coli* HB 101 and the DNA was purified from cleared lysates by CsCl/ethidium bromide density-gradient centrifugation.

Restriction Enzymes and Gel Electrophoresis. Enzymes were purchased from New England Biolabs and used as recommended by the manufacturer. Cleaved DNAs were electrophoresed on horizontal slab gels of 0.7% agarose in Tris borate buffer (89 mM Tris borate/89 mM boric acid/2 mM EDTA) containing 0.5 μg of ethidium bromide per ml at 25 V for 15 hr. The DNA was visualized with long-wave UV light. DNA-containing agarose bands were electroeluted and extracted with phenol/chloroform/isoamyl alcohol.

Establishment of Immortal/Tumorigenic Lines. *Hin*dIII-linearized plasmid DNAs (*Hin*dIII cuts once within pBR322) were mixed with 5 μg of salmon sperm DNA and precipitated with calcium phosphate, and aliquots (0.5 ml) were applied to subconfluent monolayers of normal SHE cells (passage 4; 2 × 10^5 cells per 60-mm dish) (3). Cultures were refed fresh medium at 4 hr after transfection and were serially passaged (1:10 or 1:20 split ratio) at confluency. Phenotypic alterations associated with transformation were determined as described (3, 7). Colonies in 0.3% agarose were counted at 3 wk and scored as medium (0.1–0.2 mm) or large (> 0.2 mm) by using a calibrated microscope eyepiece. Cloning efficiency (CE) was expressed as the (number of colonies × 100)/number of seeded cells. Tumorigenic potential was determined in newborn (1–3 days) Syrian hamsters injected s.c. with 2 × 10^6 cells.

Antibodies. Monospecific anti-ICP 10 serum was prepared by immunizing BALB/c mice with ICP 10 purified to

Abbreviations: HSV-2, herpes simplex virus type 2; HSV-1, herpes simplex virus type 1; SHE, Syrian hamster embryo; m.u., map unit(s); kb, kilobase(s); CE, cloning efficiency; PSTP, post-second treatment passage; mAb, monoclonal antibody; ICP, infected cell protein.

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radiochemical homogeneity (8). Monoclonal antibody (mAb) 48S was the gift of M. Zweig (National Cancer Institute, Frederick, MD). It recognizes type common determinants on herpes simplex virus type 1 (HSV-1) ICP 6 protein (9) and precipitates both HSV-1- and HSV-2-induced ribonucleotide reductase (5). mAb 20αD4, which precipitates glycoprotein gB from HSV-1- and HSV-2-infected cells (10), was provided by W. Rawls (McMaster University, Ontario, Canada). mAb Mycl-6E10 was prepared in mice immunized with a synthetic peptide consisting of 18 amino acids within a region [amino acids 171–188 (11)] of the human p64/67$^{c\text{-}myc}$ that is similar to a sequence in the mouse gene product (27).

mRNA Selection and Cell-Free Translation. Total cytoplasmic RNA was extracted at 8 hr after infection of HEp-2 cells with 20 plaque-forming units of HSV-2 per cell (12). Heat-denatured plasmid DNA was immobilized on nitrocellulose filters and used to select complementary mRNA. DNA fragments generated by endonuclease digestion of pGH17a (Fig. 1) were denatured in alkali, transferred to nitrocellulose filters (13), and used to select mRNA. Hybrid-selected mRNA was translated *in vitro* in a micrococcal nuclease-treated rabbit reticulocyte lysate (12). The translation products were labeled with [^{35}S]methionine and were analyzed by $NaDodSO_4$/PAGE.

Immunoprecipitation and $NaDodSO_4$/PAGE. Immunoprecipitation, electrophoretic separation of radiolabeled proteins on bisacrylamide cross-linked gels, and autoradiography were performed as described (8, 9). Using this procedure, we originally estimated the molecular mass of ICP 10 at 160 kDa ±10%. However, a more recent estimate obtained on *N,N'*-diallyltartardiamide-cross-linked gels was 144 kDa (unpublished data) and is consistent with the nomenclature of others (5, 14). Similar differences in estimated molecular masses in this region were reported by other laboratories using these two different cross-linked gels (15).

Tryptic Fingerprint Analysis. The 144-kDa protein translated *in vitro* under the direction of hybrid-selected mRNA was precipitated with mAb 48S. The 144-kDa protein from HSV-2-infected cell extracts was precipitated by anti-ICP 10 serum. The precipitates were electrophoresed on $NaDodSO_4$/polyacrylamide gels. Bands were identified by fluorography, excised, soaked in 0.125 M Tris·HCl, pH 6.8/0.1% $NaDodSO_4$/1 mM EDTA for 30 min, and placed in the sample wells of a second 12.5% $NaDodSO_4$/polyacrylamide gel. Chymotrypsin (1 mg/ml) in the same buffer containing 1% glycerol was added and electrophoresis was performed (16).

DNA Hybridization Analysis. High molecular weight cellular DNA was extracted from transformed cells as described (17). It was cleaved with *Bam*HI, electrophoresed through agarose gels, denatured, and transferred to nitrocellulose filters according to the method of Southern (13). Hybridization was carried out at 68°C for 15 hr with nick-translated, ^{32}P-labeled probe [pGH17a (Fig. 1); specific activity, 1–2 × 10^8 cpm/μg] in buffer containing 0.95 M NaCl, 60 mM Tris·HCl, 3 mM EDTA, 0.05% $NaDodSO_4$, 10× concentrated Denhardt's reagent (1× = 0.02% each of Ficoll 400, bovine serum albumin, and polyvinylpyrrolidone), and 150 μg of heat-denatured salmon sperm DNA per ml. After hybridization, the filters were washed twice each with 0.5 M NaCl/60 mM Tris·HCl buffer for 1 hr and 0.3 M NaCl/40 mM Tris·HCl buffer for 30 min at 68°C and once in 0.3 M NaCl/40 mM Tris·HCl buffer for 5 min at room temperature. The filters were air-dried and exposed to Kodak XAR film with intensifying screen at −70°C.

RESULTS

Immortalization and Tumorigenic Potential. We have shown that cultures transfected with fragment *Eco*RI/*Hin*dIII AE (0.419–0.525 m.u.) escape senescence but are not tumorigenic (3), suggesting that sequences within the right-hand 36% of *Bgl* II C (0.525–0.580 m.u.) [now represented by the *Bam*HI E fragment cloned as pGH17a (0.533–0.584 m.u.)] may be required for conversion to a neoplastic phenotype. Consistent with these previous findings, a nonsenescent cell line was established by transfection of SHE cells with 0.5 μg of linearized pGR75 (*Eco*RI/*Hin*dIII AE) and was designated AE. Since we could not obtain a nonsenescent cell line by transfection of SHE cells with linearized pGH17a (*Bam*HI E), AE cells (posttreatment passage 51) were transfected with subfragments (4.4, 2.0, and 1.0 kb) generated by *Bam*HI/*Sac* I digestion of pGH17a (Fig. 1) mixed with salmon sperm DNA (5 μg). The passage number after this transfection is expressed as post-second treatment passage (PSTP). At PSTP 12–14, AE cells transfected with 0.5 and 5.0 μg of the 4.4-kb subfragment of *Bam*HI E (0.554–0.584 m.u.) manifested morphological alterations consisting of refractile, spindle-shaped, rapidly dividing cells that grew with a criss-crossed growth pattern (Fig. 2*B*). Similar changes were not observed in AE cultures transfected with 0.1 or 0.5 μg of a mixture (2:1) of the 2.0-kb (0.533–0.546 m.u.) and 1.0-kb (0.546–0.553 m.u.) subfragments or with 5 μg of pBR322 or with salmon sperm DNA alone (Fig. 2*A*).

Phenotypic Characteristics of Transformed Lines. The following cultures were studied: AE cells transfected with salmon sperm DNA; E6 derived from the transfection of AE cells with the 4.4-kb subfragment of *Bam*HI E; E6C1 derived from an agarose colony of E6; E3 obtained from AE cultures transfected with the 2.0-kb and 1.0-kb subfragments (Table 1). E6 and E6C1 exhibited 4- to 5-fold higher saturation densities than AE and E3, a high CE in 2% serum (7.5–8.3%) when seeded at low cell density (200 cells per 60-mm dish), and colony formation in 0.3% agarose (0.2–2.6% CE). E6 gave rise to a mixture of medium and large agarose colonies; E6C1 developed predominantly large colonies. The E6 and E6C1 lines were highly tumorigenic (incidence 8/8 and 11/11, respectively, at 4–5 wk after inoculation), causing invasive metastatic anaplastic fibrosarcomas. AE and E3 cells did not clone in agarose and were not tumorigenic. Normal SHE cells did not exhibit any phenotypic changes.

Protein(s) Encoded by 4.4-kb Subfragment. We used *in vitro* translation directed by hybrid-selected mRNA to identify protein(s) encoded by the 4.4-kb subfragment (0.554–0.584 m.u.). Cell-free translation of mRNA selected from HSV-2-infected cells is shown in Fig. 3. The 4.4-kb subfragment

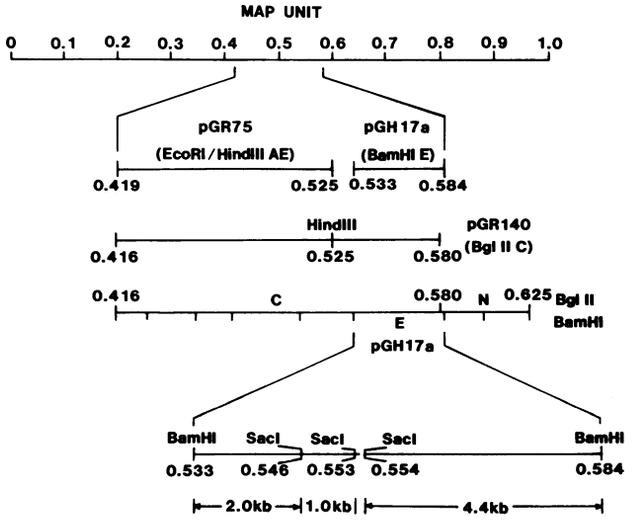

FIG. 1. Physical map of HSV-2 DNA fragments within the 0.416–0.625 m.u. region.

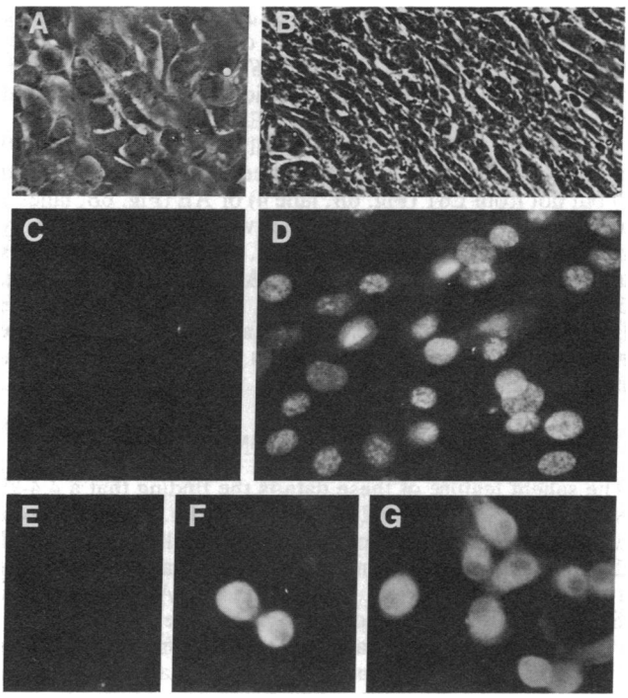

FIG. 2. Phase-contrast micrographs of AE cells (*A*) and E6C1 cells (*B*). (×100.) Indirect immunofluorescent staining with mAb 48S: AE cells (*C*) and E6C1 cells (*D*). Indirect immunofluorescent staining with mAb Mycl-6E10: E6C1 cells (*E*) and E6T cells (*F* and *G*). (×400.) AE and E6C1 at PSTP 21–25; E6T at passage 3.

selected mRNA for 144-kDa (major species) and 38-kDa (minor species) proteins (Fig. 3, lane 1). These proteins were not observed when the 1.0- and 0.2-kb subfragments (0.546–0.554 m.u.) were used for mRNA selection (Fig. 3, lane 2) or when mRNA was selected with pBR322 DNA (Fig. 3, lane 3). pGR140 (*Bgl* II C) also selected mRNA for the 144-kDa but not for the 38-kDa protein (Fig. 3, lane 4). Independent studies, including sequence analyses (12, 18, 19), suggest that mRNA for the 38-kDa protein is selected by the 4.4-kb subfragment through hybridization to the 0.55-kb region (0.580–0.584 m.u., Fig. 1) that overlaps with *Bgl* II N.

To further characterize the 144-kDa protein, products of *in vitro* translation and extracts from HSV-2-infected cells were precipitated with various antibodies. As shown in Fig. 4*A*, the 144-kDa protein was precipitated from translation products of pGH17a-selected mRNA with mAb 48S (Fig. 4*A*, lane 2) and with anti-ICP 10 serum (Fig. 4*A*, lane 1) but not with mAb 20αD4 (Fig. 4*A*, lane 3). The 144-kDa protein was also precipitated by anti-ICP 10 serum from translation products of pGR140-selected mRNA (Fig. 4*A*, lane 4) and by mAb 48S

Table 1. Phenotypic alterations induced by *Bam*HI E subfragments

Cell line	PSTP	Saturation density ($\times 10^{-5}$)*	% CE 10% serum	% CE 2% serum	% CE Agarose	Tumorigenicity†
AE	21	10.3	16.5	1.2	<0.001	0/7
E3	21	8.8	12.9	1.0	<0.001	0/12
E6	21	44.5	20.5	7.5	0.2	8/8
E6C1	25‡	50.5	47.0	8.3	2.6	11/11
Normal SHE	4§	9.8	<0.1	<0.1	<0.001	0/6

*Number of cells per 25-cm² culture flask.
†Number of tumor-bearing animals/number of inoculated animals.
‡Passage 4 after isolation of the agarose colony.
§Passage.

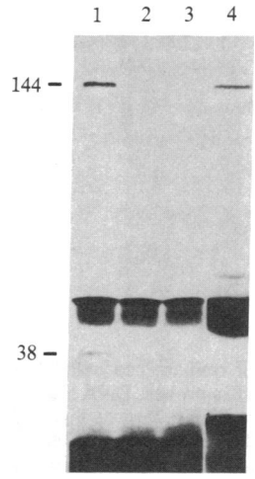

FIG. 3. Cell-free translation of HSV-2 mRNA hybrid-selected with 4.4-kb DNA (lane 1), 1.0- and 0.2-kb DNA (lane 2), pBR322 DNA (lane 3), and pGR140 (lane 4) and translated in a reticulocyte lysate system. Molecular masses are given in kDa.

(Fig. 4*A*, lane 6) and anti-ICP 10 serum (Fig. 4*A*, lane 5) from HSV-2-infected cells. Glycoprotein gB (120 kDa) precipitated with mAb 20αD4 is shown as control (Fig. 4*A*, lane 7). Tryptic peptide analysis (Fig. 4*B*) indicates that the 144-kDa protein precipitated by mAb 48S from the translation products directed by the pGR140-selected mRNA (lane 2) has a very similar peptide composition to the protein precipitated from the infected cells by anti-ICP 10 serum (Fig. 4*B*, lane 1). Although differences were observed in the intensity of some bands, all peptides were present in both preparations, thereby establishing their structural relationship.

DNA Homologous to *Bam*HI E in Transformed Cells. DNA extracted from the AE and E6C1 lines was digested with *Bam*HI, electrophoresed through a 0.7% agarose gel, and analyzed by Southern transfer hybridization with ^{32}P-labeled probes (Fig. 5). Under the relatively stringent conditions of hybridization used in these series, hybridization of the ^{32}P-labeled pGH17a probe (Fig. 5*A*) to *Bam*HI/*Sac* I-cleaved pGH17a revealed two hybridizing bands (4.4 and 2.0 kb) (Fig. 5*A*, lane 1), consistent with expected products (Fig. 1). Two other expected bands (1.0 and 0.2 kb) electrophoresed out of the gel. Under the same hybridization conditions, five discrete (5.6, 5.0, 3.8, 3.5, and 1.8 kb) bands were detected in E6C1 (Fig. 5*A*, lane 3) but not in AE (Fig. 5*A*, lane 2) cells.

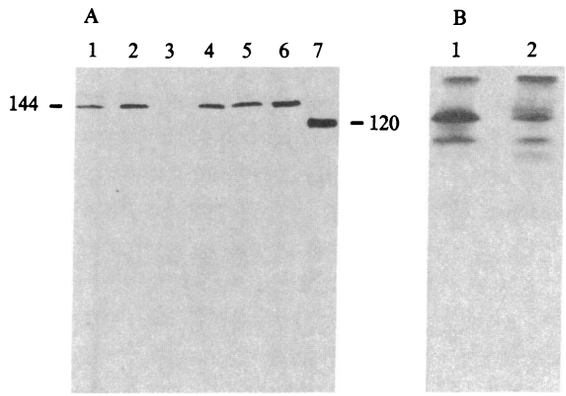

FIG. 4. (*A*) Cell-free translation products of mRNA hybrid-selected with pGH17a were precipitated with anti-ICP 10 serum (lane 1), mAb 48S (lane 2), and mAb 20αD4 (lane 3). Translation products of pGR140-selected mRNA were precipitated with anti-ICP 10 serum (lane 4). Extracts of HSV-2-infected cells were precipitated with anti-ICP 10 serum (lane 5), mAb 48S (lane 6), and mAb 20αD4 (lane 7). (*B*) Tryptic fingerprint analysis of the 144-kDa protein precipitated by anti-ICP 10 serum from an extract of infected cells (lane 1) and the 144-kDa protein precipitated by mAb 48S from cell-free translation products of pGR140-selected mRNA (lane 2). Molecular masses are given in kDa.

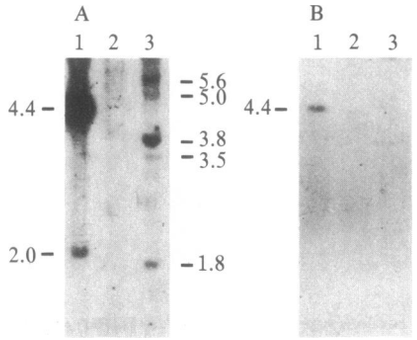

FIG. 5. Southern blot hybridization of transformed cell DNA with ^{32}P-labeled pGH17a (*A*) or pBR322 (*B*) as probes. DNA (10 μg) from AE (lanes 2) and E6C1 (lanes 3) cells (PSTP 25) was digested with *Bam*HI and electrophoresed on a 0.7% agarose gel. Reconstruction experiments, in which 5×10^{-5} μg of *Bam*HI/*Sac* I-cleaved pGH17a and 5×10^{-5} μg of pBR322 were mixed with 10 μg of salmon sperm DNA, are shown in lanes 1. In the *A* reconstruction, the 4.4-kb region represents hybridization with both pBR322 and the 4.4-kb subfragment of *Bam*HI E. Molecular sizes are indicated in kb.

When ^{32}P-labeled pBR322 was used as probe (Fig. 5*B*), neither AE (Fig. 5*B*, lane 2) nor E6C1 (Fig. 5*B*, lane 3) cells showed detectable hybridizing bands (reconstruction is shown in Fig. 5*A*, lane 1).

Proteins Expressed in Transformed Cells. Two mAbs were studied: (*i*) mAb 48S, which specifically recognizes the 144-kDa protein from HSV-2-infected cells (Fig. 4*A*), and (*ii*) mAb Myc1-6E10, which recognizes p64/67$^{c\text{-}myc}$, as established by Evan *et al.* (27) according to criteria used previously for rabbit antisera against this synthetic peptide (20). The specificity of mAb Myc1-6E10 was confirmed in our laboratory (Fig. 6*A*); it precipitated a 67-kDa protein (Fig. 6*A*, lane 1) from extracts of K562 cells [which synthesize c-*myc* protein (21)], whereas this protein was not precipitated by nonimmune serum (Fig. 6*A*, lane 2).

AE, E6C1, and a tumor derivative established with E6C1 at PSTP 30 (E6T) were stained with the two mAbs in indirect immunofluorescence (7). AE cells did not stain with either antibody (Fig. 2*C*). E6C1 cells evidenced nuclear staining with mAb 48S (Fig. 2*D*), but they did not stain with mAb Myc1-6E10 (Fig. 2*E*). In contrast, E6T cells did not stain with mAb 48S, but ≈25% of the cells evidenced cytoplasmic and nuclear staining with mAb Myc1-6E10 (Fig. 2 *F* and *G*).

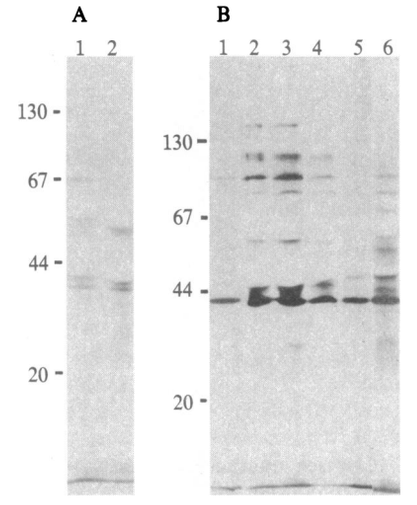

FIG. 6. Immunoprecipitation of [^{35}S]methionine-labeled proteins. (*A*) Extract of K562 cells with mAb Myc1-6E10 (lane 1) or normal rabbit serum (lane 2). (*B*) Extract of E6C1 cells (PSTP 25) with normal mouse serum (lane 1) or with mAb 48S (lane 2), extract of E6C1 cells (PSTP 35) with mAb 48S (lane 3), extract of E6T cells (passage 5) with mAb 48S (lane 4), and extract of AE cells (PSTP 25) with mAb 48S, 100 μg of protein (lane 5) and 500 μg of protein (lane 6). Molecular masses are given in kDa.

A 144-kDa protein was specifically precipitated (Fig. 6*B*) by mAb 48S from extracts of E6C1 cells (Fig. 6*B*, lanes 2 and 3) but not from E6T (Fig. 6*B*, lane 4) or AE (Fig. 6*B*, lanes 5 and 6) cells. The 144-kDa protein was not precipitated by nonimmune serum (Fig. 6*B*, lane 1). A 120-kDa protein was also specifically precipitated by mAb 48S from E6C1 (Fig. 6*B*, lanes 2 and 3) and E6T (Fig. 6*B*, lane 4) but not from AE (Fig. 6*B*, lanes 5 and 6) cells. Some additional proteins were nonspecifically precipitated by both the 48S and the nonimmune serum from all three lines.

DISCUSSION

The salient feature of these data is the finding that a 4.4-kb subfragment of *Bam*HI E (0.554–0.584 m.u.) that encodes a 144-kDa protein antigenically and structurally similar to ICP 10 [identified as one component of the viral ribonucleotide reductase (5)] converts *Eco*RI/*Hin*dIII AE-immortalized SHE cells to a fully tumorigenic phenotype.

Several studies indicate that escape from senescence (immortalization) is an early event in neoplastic transformation of rodent cells *in vitro*, which can be mediated by viral genes other than those required for the full expression of a tumorigenic phenotype. In the case of adenovirus and polyoma virus, it was shown that one class of genes (E1A and large T, respectively) is required for escape from senescence, whereas a second class (E1B and middle T) is required for the full expression of the tumorigenic phenotype (22, 23).

In accord with these observations, we found that SHE cells transfected with the *Eco*RI/*Hin*dIII AE fragment (0.419–0.525 m.u.) grew into an established but not tumorigenic line (AE). Transfection of the immortalized AE cells with a 4.4-kb subfragment of *Bam*HI E (0.554–0.584 m.u.) gave rise to lines displaying a fully transformed phenotype, including high saturation density, high CE in 2% serum, anchorage-independent growth, and metastatic tumor formation in 100% of inoculated animals. AE cells transfected with the 2.0-kb (0.533–0.546 m.u.) and 1.0-kb (0.546–0.553 m.u.) subfragments of *Bam*HI E did not acquire anchorage-independent growth and tumorigenic potential. These findings demonstrate that sequences within the 4.4-kb subfragment are specifically involved in imparting these properties. However, it is of interest to investigate whether the 4.4-kb subfragment can be further reduced without loss of tumorigenic potential. Significantly, neoplastic transformation of immortalized rat cells by the *Bam*HI E fragment was independently reported by Jariwalla *et al.* (28).

Two proteins (144 and 38 kDa) were specified by mRNA selected from HSV-2-infected cells with the 4.4-kb subfragment of *Bam*HI E. Based on immunoprecipitation and tryptic peptide studies (Fig. 4), the 144-kDa protein was identified as ICP 10, a protein found previously in HSV-2-transformed cells (1, 7) and tentatively identified as a component of the viral ribonucleotide reductase (5). Although Galloway *et al.* (12) originally suggested that the reading frames for the 144- and 38-kDa proteins are at least partially colinear, more recent sequence analysis data (18, 19) indicated that the coding regions of these two proteins do not overlap, and there is no homology in the amino acid sequence of the two proteins as determined by computer analysis (18). Based on these findings, and on our failure to select mRNA for the 38-kDa protein with the *Bgl* II C fragment (pGR140) (Fig. 3), we conclude that the mRNA for the 38-kDa protein is selected by the 4.4-kb subfragment through hybridization to the 0.55-kb region that overlaps with *Bgl* II N (0.580–0.584 m.u.). This

region encodes the carboxyl terminus of the 144-kDa protein and the transcriptional regulatory sequences (but not the amino acid sequence) for the 38-kDa protein (18, 19). Our observation that mAb 48S recognizes only the 144-kDa protein from HSV-2-infected cells is consistent with these interpretations and with the previous findings of Showalter *et al.* (9). It should be noted that the *Bgl* II N fragment does not transform normal diploid hamster cells in the continuous passage assay (3), though it causes focus formation of the NIH 3T3 aneuploid cell line and of normal rat embryo cells (2, 4).

The fate of viral DNA sequences in the transformed cells remains unclear. Thus, five hybridizing bands homologous to the pGH17a probe and ranging from 5.6 kb to 1.8 kb were detected in the *Bam*HI-cleaved E6C1 DNA but not in AE DNA. These bands are not homologous to pBR322 DNA, since they were not detected when pBR322 DNA was used as a probe. If the 4.4-kb subfragment was incorporated into cellular DNA, *Bam*HI digestion would be expected to give rise to bands of a molecular size greater than 4.4 kb. Though the exact identity of the homologous bands remains to be established, it may be significant that two of the five hybridizing bands fall into this category. Possibly, the 4.4-kb subfragment is integrated into the host cell genome, and portions of the fragment are deleted by excision or rearrangement of these sequences. Alternatively, the homologous bands represent amplified cellular sequences with homology to the *Bam*HI E fragment. Cloning of the homologous DNA sequences detected in the E6C1 cells together with flanking cellular sequences should provide more information pertaining to the identity of the homologous bands.

Huszar and Bacchetti (14) proposed that alterations in the regulatory control of ribonucleotide reductase may cause mutations in cellular DNA. We have no direct evidence to indicate that the 144-kDa protein or the ribonucleotide reductase activity is involved in transformation mediated by the 4.4-kb subfragment. However, immunofluorescence and immunoprecipitation studies with mAb 48S suggest that, at least up to PSTP 35, E6C1 cells express a protein antigenically similar to the infected cell 144-kDa protein. This protein is not present in AE cells nor in the tumor derivative E6T. The reason for the presence of lower molecular mass proteins in the precipitates obtained from transformed cell extracts is not clear. However, most of these proteins are also precipitated from all extracts by normal mouse serum, suggesting that they represent nonspecific aggregation. The exception is a 120-kDa protein that is not observed in AE cells and is specifically precipitated by mAb 48S from E6C1 and E6T cells. The relationship, if any, between the 144- and 120-kDa proteins is unclear. Further studies, including (*i*) comparative peptide mapping of the two proteins and (*ii*) the presence or absence of authentic sequences homologous to the 4.4-kb subfragment in the E6T cells that express the 120-kDa protein, should help resolve this question. Preliminary data (unpublished) suggest that sequences homologous to the pGH17a probe are present in E6T cells. However, their state remains to be established. In any case, the entire 144-kDa protein is probably not required for transformation, since the latter is also mediated by the *Bgl* II C fragment (3) that does not encode the carboxyl terminus of the 144-kDa protein (18, 19). Possibly, the 144-kDa protein contains different functional domains, such that the enzymatic activity and/or transforming potential may be located at different or similar sites. Indeed, functionally different truncated forms of the large tumor antigen of simian virus 40 have been identified in transformed cells (24), and only the amino-terminal 45 kDa of the v-*abl*-encoded 130-kDa protein is required for fibroblast transformation by Abelson murine leukemia virus (25).

It may be significant that E6T, the tumor derivative established with the transformed cells, is positive for c-*myc* oncogene expression in 25% of the cell population, although the transformed E6C1 cells were negative before their growth in animals. At present, the role of c-*myc* expression in neoplastic transformation induced by the 4.4-kb subfragment remains unclear. It is tempting to speculate that the expression of domains of the 144-kDa protein encoded within *Bgl* II C is responsible for initiation of c-*myc* oncogene transcription, since ribonucleotide reductase has been associated with a mutator phenotype (26).

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1. Jariwalla, R. J., Aurelian, L. & Ts'o, P. O. P. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2279–2283.
2. Galloway, D. A. & McDougall, J. K. (1981) *J. Virol.* **38**, 749–760.
3. Jariwalla, R. J., Aurelian, L. & Ts'o, P. O. P. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5902–5906.
4. Cameron, I. R., Park, M., Dutia, B. M., Orr, A. & Macnab, J. C. M. (1985) *J. Gen. Virol.* **66**, 517–527.
5. Bacchetti, S., Evelegh, M. J., Muirhead, B., Sartori, C. S. & Huszar, D. (1984) *J. Virol.* **49**, 591–593.
6. Lozzio, C. B. & Lozzio, B. B. (1975) *Blood* **45**, 321–334.
7. Manak, M. M., Aurelian, L. & Ts'o, P. O. P. (1981) *J. Virol.* **40**, 289–300.
8. Strnad, B. C. & Aurelian, L. (1978) *Virology* **87**, 401–415.
9. Showalter, S. D., Zweig, M. & Hampar, B. (1981) *Infect. Immun.* **34**, 684–692.
10. Balachandran, N., Bacchetti, S. & Rawls, W. (1982) *Infect. Immun.* **37**, 1132–1137.
11. Colby, W. W., Chen, E. Y., Smith, D. H. & Levinson, A. D. (1983) *Nature (London)* **301**, 722–725.
12. Galloway, D. A., Goldstein, L. C. & Lewis, J. B. (1982) *J. Virol.* **42**, 530–537.
13. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
14. Huszar, D. & Bacchetti, S. (1983) *Nature (London)* **302**, 76–79.
15. Pereira, L., Wolfe, M. H., Fenwick, M. & Roizman, B. (1977) *Virology* **77**, 733–749.
16. Cleveland, D., Fischer, S., Kirschner, M. & Laemmli, V. (1977) *J. Biol. Chem.* **252**, 1102–1106.
17. Gross-Bellard, M., Oudet, P. & Chambon, P. (1973) *Eur. J. Biochem.* **36**, 32–38.
18. McLauchlan, J. & Clements, J. B. (1983) *EMBO J.* **2**, 1953–1961.
19. Galloway, D. A. & Swain, M. A. (1984) *J. Virol.* **49**, 724–730.
20. Ramsay, G., Evan, G. I. & Bishop, J. M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7742–7746.
21. Hann, S. R., Thompson, C. B. & Eisenman, R. N. (1985) *Nature (London)* **314**, 366–369.
22. Van den Elsen, P. J., Houweling, A. & Van der Eb, A. J. (1983) *Virology* **128**, 377–390.
23. Rassoulzadegan, M., Cowie, A., Carr, A., Glaichenhaus, N., Kamen, R. & Cuzin, F. (1982) *Nature (London)* **300**, 713–718.
24. Chaudry, F., Harvey, R. & Smith, A. E. (1982) *J. Virol.* **44**, 54–66.
25. Prywes, R., Foulkes, J. G., Rosenberg, N. & Baltimore, D. (1983) *Cell* **34**, 569–579.
26. Weinberg, G., Ullman, B. & Martin, D. W., Jr. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2447–2451.
27. Evan, G. I., Lewis, G. K., Ramsay, G. & Bishop, J. M. (1985) *Mol. Cell. Biol.*, in press.
28. Jariwalla, R. J., Tanczos, B., Jones, C., Ortiz, J. & Salimi-Lopez, S., *Proc. Natl. Acad. Sci. USA*, in press.