Multiple Copies of Shope Virus DNA Are Present in Cells of Benign and Malignant Non-Virus-Producing Neoplasms

JACK G. STEVENS* AND FELIX O. WETTSTEIN

Department of Microbiology and Immunology, School of Medicine, University of California, Los Angeles, Los Angeles, California 90024

In initial efforts to characterize the virological basis of neoplasia in the Shope papilloma-carcinoma system, the extent to which the viral genome is present in non-virus-producing benign and malignant tumors in domestic rabbits was established. Employing nick-translated radioactive viral DNA purified from productively infected papillomas on cottontail rabbits as a probe, it was found that (i) papillomas, primary carcinomas, and metastatic carcinomas contain 10 to about 100 copies of the viral genome per diploid cell equivalent of DNA and (ii) viral DNA is present in detectable amounts in essentially all neoplastic cells. These results are consistent with the suggestion that continued presence of the viral genome is necessary for induction and maintenance of malignant as well as benign neoplasms.

In Eastern cottontail rabbits (*Sylvalagus floridanus*) the Shope papilloma virus induces virus-producing papillomas and, in some instances, carcinomas (non-virus-producing) appear at the same site several months later. A similar two-stage course of events occurs in domestic rabbits (*Oryctalagus cuniculus*), but in these animals, neither tumor characteristically produces virus, and a greater proportion of papillomas become malignant (11, 18). Although the agent is not present in the latter tumors (or present at very low levels in some instances), viral genetic information is conserved, since DNA extracted from either induces the same papilloma-carcinoma sequence when introduced into the skin of other domestic rabbits (9, 10, 11). In addition, it has been reported that virus-specific antigens are detectable in cells of papillomas and in tissue cultures derived from them (15, 17, 20).

Obviously, this system has considerable potential for studies of virus-induced neoplastic events since it represents a two-stage process, apparently involving the continued presence and expression of viral genetic information in epidermal cells. Although the agent cannot be replicated in cell culture, suitable methods of radioactive labeling that uses viral products obtained from productively infected cottontail-derived papillomas are now available to further define and exploit this system. By using these techniques, we have initiated systematic studies in the animal. Our initial investigations concern qualitative and quantitative determinations of the extent to which the viral genome is present

and expressed, principally in non-virus-producing neoplasms. In this communication, through the application of liquid-phase molecular reassociation experiments, we show that, in all tumors, multiple copies of the viral genome are present per diploid cell quantity of cellular DNA. In addition, cytoreassociation studies demonstrate that all neoplastic cells contain viral DNA in roughly equivalent amounts.

MATERIALS AND METHODS

Animals. The wild rabbits used were either desert cottontails (*Sylvalagus audoboni*) trapped locally (by J.G.S.) or Eastern cottontail rabbits (*S. floridanus*) trapped in Kansas (E. Johnson, Rago, Kans.) or at Whidbey Island, Washington (James Thomsen, Hutchinson Cancer Center, Seattle, Wash.). Animals from the three sources were indistinguishable with respect to papilloma induction and virus replication. They were individually caged in standard rabbit cages equipped with small resting boxes and maintained on standard rabbit pellets supplemented with one-quarter apple two times a week.

Domestic rabbits of two breeds (San Juan and Polish) were also supplied by James Thomsen. These animals were selected because they have a relatively low rate of papilloma regression (J. Thomsen and C. A. Evans, personal communication) and a high proportion of the tumors become malignant.

Virus. The Washington B strain of Shope papilloma virus was kindly supplied by C. A. Evans, University of Washington, Seattle. This agent was originally isolated by Evans from a naturally occurring papilloma on a cottontail rabbit trapped at Whidbey Island (6). The agent was propagated here in cottontail rabbits and then titrated in domestic animals by methods developed by Evans and Rashad (7). Thus, a stock

virus pool titering 1.6×10^4 50% infectious doses (ID_{50}) per ml was prepared as a 10% (wt/wt) homogenized suspension in Hanks balanced salt solution, clarified by low-speed centrifugation, and stored at $-70°C$. This pool was used in all experiments. For induction of tumors to be used as a source of nucleic acids (in either rabbit genus), 300 ID_{50} of this stock virus pool in 0.2 ml were introduced into the skin of the back at four sites. Again, the methods of Evans and Rashad (7) were employed. Characteristically, papillomas appeared in 2 to 4 weeks; carcinomas appeared in 4 to 12 months. The latter tumors were tentatively classified by gross appearance, and this impression was verified by histopathological examination.

Preparation of the viral DNA probe. Viral DNA for preparation of the probe was purified from virus-producing cottontail papillomas (established by electron microscopic examination of phosphotungstic acid-stained tumor homogenates). The tumors were removed from the animal, snap-frozen in liquid nitrogen, and stored at $-70°C$. Later they were thawed, cut into small pieces and ground in a Ten Broeck tissue grinder in 0.02 M Tris buffer (pH 7.6)-0.02 M EDTA. Nucleic acids were released by incubation of this material in 5% sodium dodecyl sulfate (SDS) for 1 to 2 h at 37°C. Insoluble material was separated by low-speed centrifugation, the SDS was removed by dialysis, and the supernatant was treated with pronase at 100 μg/ml for 45 min (37°C). Supercoiled viral DNA was then separated from both linear DNA and nicked circular DNA by two cycles of CsCl (starting density 1.55 g/cm^3)-ethidium bromide (100 μg/ml) density equilibrium centrifugation (22) in a Spinco SW 50.1 rotor at 40,000 rpm for 48 h at 20°C. After removing the dye with isopropanol and the CsCl by dialysis, this DNA was further characterized as described in the next section and then nick translated (14, 24). In this procedure, [^{3}H]dCTP (20 to 30 mCi/μmol) and [^{3}H]TTP (50 to 60 mCi/μmol) were used as labeled precursors. After labeling, the radioactive DNA preparation was phenol extracted, and the trinucleotides were separated from DNA by chromatography on Sephadex G-50 in 0.14 M phosphate buffer (pH 6.8) with 0.1% SDS added. The DNA obtained was heated at 105°C for 5 min and rapidly cooled, and the intramolecular double strands (hairpins) were removed by hydroxylapaptite chromatography (26) on DNA grade hydroxylapatite (BioRad). Single strands of DNA eluted from the column were reannealed overnight at 65°C in 0.4 M phosphate buffer containing 0.3% SDS. The reannealed DNA was separated from residual single strands again by passage over hydroxylapatite, and the double strands, to be used as a probe, were collected and stored at 4°C. Probes with specific activities of 2.5×10^6 to 6.3×10^6 cpm/μg were prepared by these methods.

Electrophoretic analysis of viral DNA on agarose gels. Horizontal gel slabs (20 cm) composed of 1% agarose and 0.5 μg of ethidium bromide (27) per ml were used in these analyses. *Eco*RI and *Hin*dIII restriction endonucleases were purchased from New England Biolabs, Beverly, Md. and used at 2 U/μg of DNA (10 to 15 μl total volume, 37°C, 30 min) for digestion of Shope virus or bacteriophage λ DNA. After digestion, the endonucleases were inactivated by

heating the reaction mixture to 65°C for 5 min. The DNA samples were applied to electrophoresis gels in 0.2- to 0.5-μg quantities in 20 μl containing 25% glycerol and moved into the gel for 1 h at 25 V. The sample slots were then filled with agarose, the gel was covered with a plastic sheet, and the electrophoresis was continued for 14 to 16 h at 50 V. To prevent pH changes, the tank buffer was circulated.

Isolation of nucleic acids from tumors. For extraction and purification of DNA contained in the various tumors, snap-frozen specimens (again stored at $-70°C$) were ground to a fine powder in a mortar kept in dry ice. The powder was next incubated at 60°C in 0.1 M NaCl-0.01 M Tris buffer (pH 7.6), with 0.002 M EDTA, 5% SDS, and 100 U of heparin added per ml. After 3 min, 200 μg of proteinase K was added per ml and the digestion was continued for 7 min. The resulting digest was briefly sonicated to reduce viscosity, and the nucleic acids were extracted twice with phenol-chloroform-isoamyl alcohol (75:24:1) at pH 5.1 and 60°C. The nucleic acids were precipitated twice with ethanol, and DNA and RNA were separated by equilibrium centrifugation on potassium iodide gradients (29). The DNA was sonicated to a size of 4S to 6S (as determined by alkaline sucrose gradient ultracentrifugation), treated with 0.3 M NaOH at 37°C overnight, neutralized, ethanol precipitated, and stored at $-20°C$.

DNA reassociation—liquid phase. In all experiments involving tumors, the standard reannealing mixture contained a total of 2.5 mg of either tumor and/or calf thymus or rabbit liver DNA per ml in 0.4 M phosphate buffer (pH 6.8) with 0.025 M EDTA-0.1% SDS and 22,000 or 42,000 cpm/ml of Shope virus probe DNA (6.3×10^6 cpm/μg). Twenty-microliter aliquots of these mixtures were sealed in glass capillaries and the DNA was melted for 5 min at 115°C and then allowed to reanneal at 65°C in a water bath. At appropriate times, duplicate capillaries were removed from the water bath, chilled in ice, and broken, and the contents were extruded into 0.4 ml of water containing 5 μg of native calf thymus DNA. These samples were kept frozen at $-20°C$ until they were analyzed for extent of reassociation. At this time the samples were brought to 60°C and applied to 0.4 ml of hydroxylapatite columns. Single-stranded DNA was eluted with 5-column volumes of 0.14 M phosphate buffer (pH 6.8)-0.01% SDS at 60°C, and double-stranded DNA was eluted with 3-column volumes of 0.4 M phosphate buffer (also pH 6.8)-0.01% SDS and 60°C. The eluate containing double-stranded DNA was then diluted with water to 0.04 M phosphate, and 2.5 volumes of toluene Triton-X 100 scintillation fluid (21) was added to both single- and double-stranded fractions. These fractions were counted by liquid scintillation spectrometry, and the average of duplicate analyses was calculated.

Reannealing conditions for characterization of the probe were similar to those used for studies of tumor DNA. However, the reannealing mixtures contained 1 mg of yeast tRNA per ml, 0, 0.05, or 1 μg of sonicated viral DNA per ml, and 14,000 cpm of nick-translated probe DNA (2.5×10^6 cpm/μg).

Cytoreassociation. Sections of appropriate tissues were cut in a cryostat, picked up on gelatinized slides

as described earlier (4), and kept at 4°C until used. The strategy used from this point was adapted slightly from one presented by Haase et al. (8). Before annealing, the sections were rehydrated for 10 min in 2× standard saline citrate (SSC), and incubated with ribonucleases (20 µg of T1 and 200 µg of pancreatic per ml, 100 µl total volume on each section) for 1 h at 37°C. Slides were next rinsed with 0.1× SSC, and the DNA was denatured at 60°C for 2 h in 95% formamide in 0.1× SSC. After rinsing in 70 and 95% ethanol, slides were air dried, and 6,500 cpm of the probe DNA in 100 µl of 6× SSC was placed on the section, which was then overlaid with a cover slip. The nucleic acid reassociation was carried out at 66°C in a moist chamber. After 22 h, the section was rinsed several times with a buffer containing 0.03 M sodium acetate (pH 4.5), 0.1 M NaCl, and 0.002 M $ZnCl_2$, and incubated with 10 U of single-strand-specific S1 nuclease (Sigma) per ml at 50°C for 2 h. After an overnight rinse in 6× SSC, the sections were taken to 95% in ethanol, filmed with Kodak NTB-2 emulsion and stored at −20°C. After 2 to 4 weeks of exposure time the slides were developed and stained with Giemsa.

Chemical carcinogen-induced epidermal tumors. 7,12-Dimethylbenz-(α)-anthracene (DMBA)-induced papillomas were produced on the inner surface of the ear of domestic rabbits employing the method developed by Berenblum (1), using the application schedule of Rashad and Evans (23).

RESULTS

Characterization of the viral DNA probe. (i) General. If they can be obtained in sufficient quantity, purified virions are unquestionably the preferred source for viral genomic material. In our hands, however, laboratory-induced papillomas routinely yield at least an order of magnitude fewer virions than do their natural counterparts, and insufficient numbers of the latter are available for investigation. Therefore, we have purified and labeled supercoiled viral DNA extracted from infectious papillomas. In the usual case, by using the methods described in the preceding section, we have been able to recover about 10 µg of viral DNA per g of papilloma (but less than 10% of this amount can be obtained if virions are first purified). As is detailed below, such a DNA preparation is entirely adequate for these studies since: (i) it behaves as one molecular species of 5×10^6 daltons when analyzed by agarose-ethidium bromide gel electrophoresis, or if the kinetics of reassociation are determined; (ii) probe reassociation is accelerated by DNA prepared from virus-induced epidermal neoplasms, but not by similar preparations from normal rabbit liver or DMBA-induced papillomas; and (iii) in cytoreassociation studies, the probe anneals only to nuclei and, more importantly, only to nuclei of epithelial cells in virus-induced neoplasms.

(ii) Agarose gel electrophoresis. Super-

coiled DNA from CsCl gradients was analyzed for homogeneity in molecular weight in ethidium bromide-agarose gels. Before electrophoresis, the DNA was incubated with EcoRI endonuclease, an enzyme which cleaves the DNA at one site (F. O. Wettstein and J. G. Stevens, unpublished data). As molecular weight markers for construction of a calibration curve, a HindIII endonuclease digest of phage λ DNA (16) was prepared and electrophoresed in an adjoining slot. As shown in Fig. 1, the cleaved viral DNA runs as one uniform band and, from the calibration curve, a molecular weight of 5.1×10^6 can be assigned. This corresponds favorably to the molecular weight values of 4.2×10^6 to 5.5×10^6 for Shope DNA which has been obtained by others (5). Also included in the figure are slots containing uncleaved Shope virus DNA (two bands which can be identified as "nicked" and "supercoiled," respectively) and a sample of native phage DNA. Assignment of a nicked configuration to the slowly moving minor band derived from Shope DNA was based, among other considerations, on the observation that incomplete digestion with EcoRI endonuclease led to an accumulation of material with this electrophoretic mobility.

(iii) Reassociation kinetics. As a further characterization, nick-translated radioactive viral DNA was annealed in kinetic experiments with different concentrations of sonicated (7S) unlabeled viral DNA. As can be seen in Fig. 2, if percent reassociation of labeled probe DNA in the presence of excess unlabeled probe is plotted as a function of C_0t (3), the curve expected for a second-order reaction is generated. In addition, if the C_0t value for one-half maximal annealing is corrected for standard conditions, a kinetic complexity of 5×10^6 to 6×10^6 daltons can be calculated.

That the probe does not reanneal to the 100% level is due to its relatively small size (4S on alkaline sucrose gradients), and this in turn is a consequence of the methods which must be used for labeling to the high specific activity desired (a relatively high DNase concentration during nick translation). This influence of probe size on the extent of reannealing was shown when (i) single-stranded DNA remaining after extensive reassociation possessed a lower sedimentation coefficient than that which had reannealed and (ii) a higher-molecular-weight fraction separated from the standard 4S probe in alkaline sucrose gradients reannealed to a somewhat higher extent than did the parental material. For practical reasons, we were unable to routinely prepare and use this higher-molecular-weight probe. Finally, although we have not shown that nick translation labels the viral DNA uniformly, such
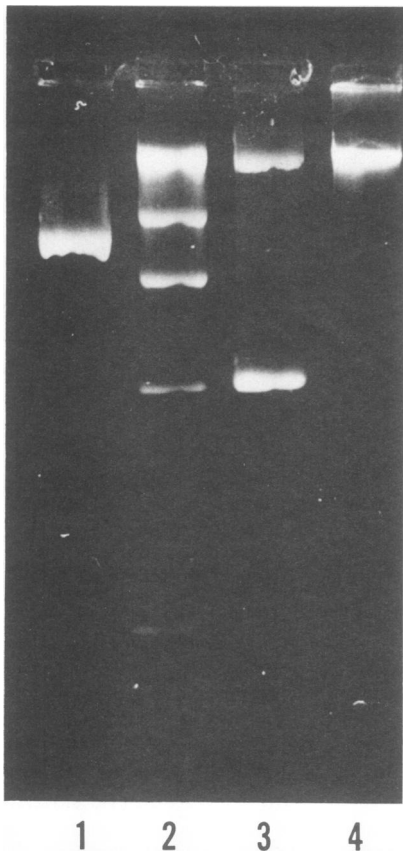
mor—papillomas from cottontail rabbits, and papillomas, primary carcinomas, and distant metastases (lung) from domestic rabbits. At least three different preparations of each tumor classification (on different animals) were analyzed, and the kinetics of reassociation of the probe in the presence of DNA from a representative of each group is presented in Fig. 3. As can be seen, DNA from all virus-induced tumors accelerated the rate of reassociation of the probe, thus indicating that all contained viral genetic information. In addition, as is discussed later, the kinetics of the annealing reactions suggest that the entire genetic complement of the virus is present. These results and those from similar experiments involving tumors from the additional animals are summarized in Table 1, where the number for viral genome equivalents per diploid cell equivalent of tumor DNA has been calculated and presented. These numbers obviously represent a minimal estimate, since the tumors are not composed solely of neoplastic cells, but, as is discussed in the next section, viral DNA appears to be limited to and present in all such cells.

Cellular distribution of viral DNA. The distribution of viral genetic information was established by cytoreassociation experiments.

FIG. 1. *Electrophoretic analysis of Shope papilloma virus DNA in ethidium bromide-agarose gels. Purified viral DNA, either undigested or cleaved with EcoRI endonuclease, was electrophoresed with molecular weight markers prepared by HindIII endonuclease digestion of bacteriophage λ DNA. Slot 1, papilloma virus DNA after digestion with EcoRI endonuclease. Slot 2, λ DNA digested with HindIII endonuclease. Slot 3, undigested papilloma virus DNA. Slot 4, undigested λ DNA. The molecular weights of the HindIII fragments of λ DNA shown in the second slot (from top to bottom) are 15×10^6, 6.4×10^6, 4.3×10^6, 2.9×10^6, 1.6×10^6, and 1.4×10^6. The presence of DNA migrating as "nicked" viral DNA in slot 3 results from the conversion of form I to form II DNA during dialysis and storage. Further interpretations and details are given in the text.*

a result seems likely since simian virus 40 DNA, when labeled by these methods, is uniformly labeled (2, 24).

Quantitative determination of viral DNA present in various tumors. In initial searches for viral DNA in the various classes of tumors, the radioactive DNA probe was employed in kinetic analyses with DNA extracted from tumors. Here, DNA to be analyzed for viral sequences was prepared from four types of tu-
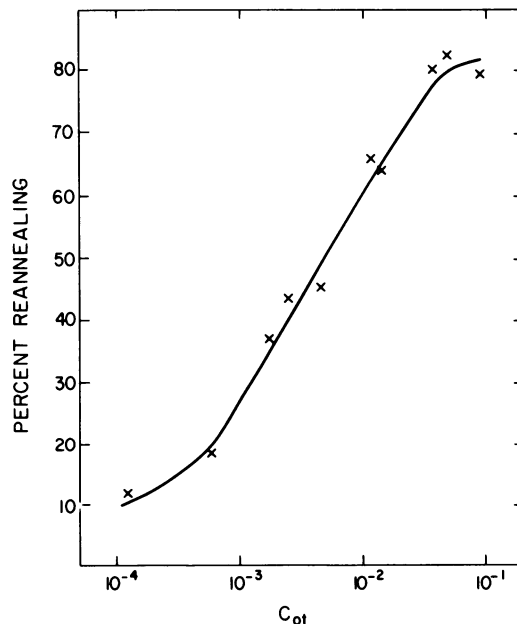

FIG. 2. *Annealing of nick-translated, radioactive Shope papilloma virus DNA with unlabeled viral DNA. Annealing experiments were performed as described in the text, and percent reannealing of the viral probe DNA was plotted as a function of C_0t (3). A further discussion is presented in the text.*
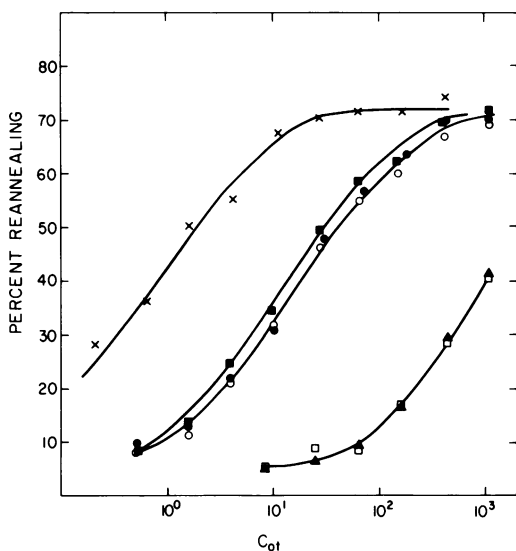
FIG. 3. *Annealing of nick-translated, radioactive Shope papilloma virus DNA to DNA extracted from Shope virus-induced tumors. Annealing experiments were performed as described in the text, and percent annealing of the probe DNA was plotted as a function of C_0t (3). The curves drawn represent annealing of the probe in mixtures containing DNA from a papilloma on a cottontail rabbit (×), DNA from a papilloma on a domestic rabbit (●), DNA from a primary carcinoma on a domestic rabbit (○), DNA from a metastatic carcinoma on a domestic rabbit (■), calf thymus DNA (□), and rabbit liver DNA (▲). It can be noted that the final extent of probe DNA reannealing in these analyses is 10% less than that shown in Fig. 2. This is most likely due to the slightly lower molecular weight of the probe DNA fragments used in these latter experiments.*

Here the probe was incubated as described in Materials and Methods with frozen sections of papillomas from both cottontail and domestic rabbits and primary carcinomas from the domestic animals. Radioautographs were prepared, and the sections were stained and examined microscopically. As is depicted in the series of photographs in Fig. 4, a concentration of silver grains was readily detectable over keratinizing cells of the virus-producing cottontail-derived papillomas (Fig. 4a, positive control), but not over any cells in the DMBA-induced papilloma from a domestic rabbit (Fig. 4b, negative control). Figure 4c shows that essentially all nuclei in neoplastic cells from a primary carcinoma on a domestic rabbit demonstrate a concentration of silver grains above the background level. Precise quantitation is impossible to achieve, but since the numbers of grains per area of nucleus does not differ greatly in most cells, it seems likely that each tumor cell nucleus harbors multiple copies of the viral genome. Results similar

to those with carcinomas were obtained with preparations made from papillomas on domestic rabbits, and in both cases grains were restricted to nuclei of epidermal cells (dermal and other cells were negative).

TABLE 1. *Number of viral genomes per diploid cell equivalent of DNA in rabbit tumors induced by Shope papilloma virus*

Animal source and tissue classification	$C_0t_{1/2}$	No. of viral genomes/ diploid cell equivalent of DNA[a]
Cottontail, papilloma		
K3	0.52	3,800
K26	0.8	2,400
K30	0.20	8,800
Domestic, papilloma		
5	25	79
10	18	81
R	125	11
Domestic, primary carcinoma		
AA	34	54
AH	20	65
CA 101	20	64
CA 102	18	72
Domestic, metastatic carcinoma		
AA	28	66
AH	20	64
AF	14	107
Domestic, DMBA-induced papilloma	1,000	<1[b]
Domestic, normal liver DNA	1,000	<1

[a] $C_0t_{1/2}$ values were derived from kinetic DNA reassociation experiments (examples depicted in Fig. 3), and the number of viral genome equivalents per diploid cell genome was calculated with the aid of a calibration curve. To establish the curve, probe DNA was reannealed in the presence of increasing amounts of unlabeled viral DNA, and the time required for half-maximal reannealing was determined at each concentration. Reciprocals of these times were plotted as a function of total viral DNA concentration, and the amount of probe plus viral DNA in the tumor DNA reassociation mixtures was determined from the calibration curve. In each calculation a correction was made for the contribution of probe DNA, and the amount of DNA per diploid rabbit cell was assumed to be 6.0×10^{-6} µg.

[b] The limits of detection are estimated to be <1 viral genome equivalent per diploid cell genome. This statement is based upon the following consideration. When 0.007 µg of probe DNA per ml is allowed to reanneal in the presence of 2.5 mg of calf thymus DNA per ml (as in Fig. 3), a "value" of two viral genomes per diploid cell equivalent of this carrier DNA can be derived. Acceleration of the reannealing reaction by one additional viral genome per diploid cell equivalent of DNA can easily be detected in this type of assay.
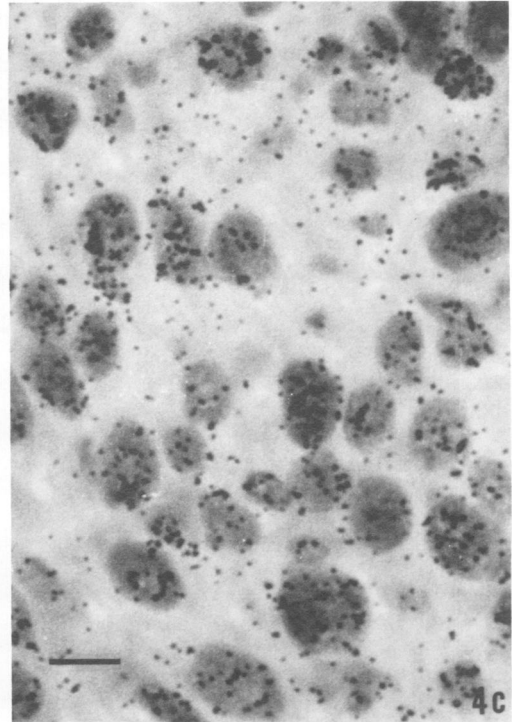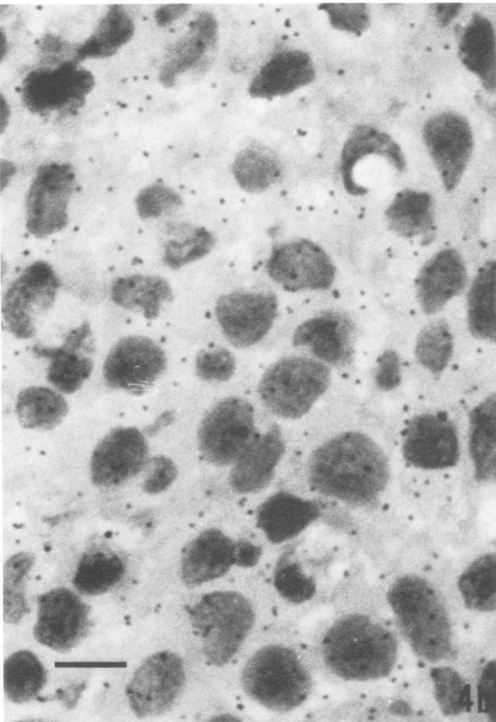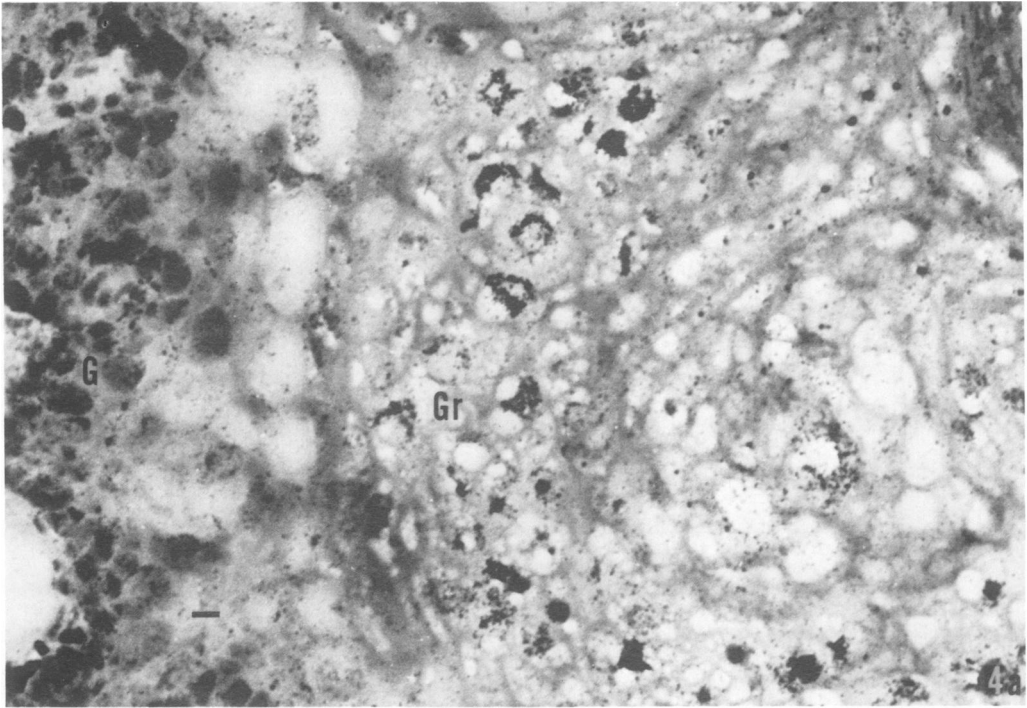
FIG. 4. *Shope papilloma virus DNA demonstrated by cytoreassociation methods in cells of a primary carcinoma from a domestic rabbit. (a) Positive control, virus-producing papilloma from a cottontail rabbit. Silver grains are selectively concentrated over individual cells and nuclei in the stratum granulosum (Gr). Melanin granules (but no concentration of silver grains) are present in the stratum germanitivum (G). (b) Negative control, DMBA-induced papilloma from a domestic rabbit. No concentration of silver grains is present over any cellular compartment. (c) Shope virus-induced primary carcinoma. Essentially all nuclei demonstrate a concentration of silver grains significantly above the background (Bar represents 10 μm). Details concerning methods and interpretations are given in the text.*

DISCUSSION

The results presented here demonstrate that 10 to approximately 100 copies of the viral genome are conserved in all epidermal cells populating benign and malignant neoplasms induced by the Shope virus in domestic rabbits. In addition, it also appears that most, if not all, of the viral DNA in these tumors represents copies of the entire viral genome. Thus, the slope of annealing curves and the final extent of annealing of the probe in reactions driven by DNA from productively or non-productively infected tumors is the same (Fig. 3). However, deletion of a small portion (10%) of genetic information from the viral DNA could not be detected by these methods, and a more definitive statement can be made if it is shown that DNA derived from the non-virus-producing tumors is capable of inducing virus-producing papillomas on cottontail rabbits. Obviously, absolute proof for presence of the entire complement of viral DNA will depend upon nucleotide sequence determination. It should be noted here that in another papilloma virus system, bovine papilloma virus genetic information is conserved in virus-induced, but apparently non-virus-producing hamster fibromas and bovine meningiomas (13).

The finding of roughly equivalent amounts of viral DNA in all neoplastic cells on domestic rabbits is of interest for at least two reasons, and these remain to be systematically investigated. First, it has been shown by others that these tumors sometimes contain small amounts of virus (6), and this is probably replicated by a few keratinizing cells in which antigens can be detected (cf. 17). However, when coupled with immunohistochemical and ultrastructural studies (15, 17; M. L. Cook, F. O. Wettstein, and J. G. Stevens, unpublished data), the results presented here suggest that the majority of cells (although obviously infected since they harbor viral DNA) are maintaining the viral genome in a latent state (defined in 28). Second, in papillomas on cottontail rabbits, viral genetic information is readily detected only in productively infected keratinizing cells (18, 19; Fig. 4a), although it has been suggested that dividing epidermal cells in the basal layers may harbor the viral genome (18). Even if the viral genome is present in these latter cells, the stage of differentiation obviously has a profound effect on viral genetic expression in cottontail rabbits. This is not the case in domestic animals, where cells in all stages of differentiation contain readily demonstrable amounts of viral DNA.

Finally, the relationship of these findings to the basic neoplastic process should be considered. Although the fundamental significance of the results are as yet unclear, they do suggest that, in domestic rabbits, the transition from normal skin to papilloma to carcinoma and maintenance of the latter state require the continuous presence of multiple and roughly equivalent numbers of viral genomes. This notion is also supported by our more recent findings which show that (i) the VX-7 and VX-2 carcinomas (originally induced by the Shope virus in the skin of domestic rabbits and subsequently transplanted serially in rabbits for over 25 [25] and 35 years [12], respectively) also contain multiple genome equivalents of viral DNA per diploid cell quantity of DNA, and (ii) the viral genome is transcribed to a limited extent in both benign and malignant neoplasms in domestic rabbits. Investigations concerning the physical state of the viral genome in the neoplastic cells and pertinent characterization of the products of viral genetic expression are now underway. These studies should give important insight into the precise role played by the virus in these transitions.

LITERATURE CITED

1. **Berenblum, I.** 1945. 9,10-Dimethyl-1,2-Benzanthracene as a highly potent carcinogen for the rabbit's skin. Cancer Res. **5**:265–268.
2. **Botchin, M., W. Topp, and J. Sambrook.** 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell **9**:269–287.
3. **Britten, R. J., and D. E. Kohne.** 1968. Repeated sequences in DNA. Science **161**:529–540.
4. **Cook, M. L., V. B. Bastone, and J. G. Stevens.** 1974. Evidence that neurons harbor latent herpes simplex virus. Infect. Immun. **9**:946–951.
5. **Crawford, L. V.** 1969. Nucleic acids of tumor viruses. Adv. Virus Res. **14**:89–152.
6. **Evans, C. A., and A. L. Rashad.** 1965. Variations in amount of virus and in microscopic structure of Shope papillomas of cottontail rabbits, p. 187–208. *In* M. Pollard (ed.), Perspectives in virology, vol. 4. Harper and Row, New York.
7. **Evans, C. A., and A. L. Rashad.** 1967. Virus content of Shope papillomas of cottontail rabbits. Cancer Res. **27**: 1011–1015.
8. **Haase, A. T., L. Stowring, O. Narayan, D. Griffin, and D. Price.** 1977. Slow persistent infection caused by Visna virus. Role of host restriction. Science **195**:175–177.
9. **Ito, Y.** 1962. Relationship of components of papilloma virus to papilloma and carcinoma cells. Cold Spring Harbor Symp. Quant. Biol. **27**:387–394.
10. **Ito, Y.** 1963. Studies on subviral tumorigenesis: carcinoma derived from nucleic acid-involved papillomas of rabbit

skin. Acta Unio Int. Contra Cancrum 19:280–283.

11. **Ito, Y.** 1975. Papilloma-myxoma viruses, p. 323–340. *In* E. F. Becker (ed.), Cancer, vol. 2. Plenum Press, New York.

12. **Kidd, J. G., and P. Rous.** 1940. A transplantable rabbit carcinoma originating in a virus-induced papilloma and containing the virus in a masked or altered form. J. Exp. Med. 71:813–838.

13. **Lancaster, W. D., C. Olson, and W. Meinke.** 1976. Quantitation of bovine papilloma viral DNA in viral-induced tumors. J. Virol. 17:824–831.

14. **Maniatis, T., A. Jeffrey, and D. Kleid.** 1975. Nucleotide sequence of the rightword operator of phage λ. Proc. Natl. Acad. Sci. U.S.A. 72:1184–1188.

15. **Mellors, R. C.** 1960. Tumor cell localization of the antigens of the Shope papilloma virus and the Rous sarcoma virus. Cancer Res. 20:774–753.

16. **Murray, E., and N. E. Murray.** 1975. Phage lambda DNA receptor chromosomes for DNA fragments made with restriction endonuclease III of *Haemophilus influenzae* and restriction endonuclease I from *Escherichia coli.* J. Mol. Biol. 98:557–564.

17. **Noyes, W. F., and R. C. Mellors.** 1957. Fluorescent antibody detection of the antigens of the Shope papilloma virus in papillomas of the wild and domestic rabbit. J. Exp. Med. 106:555–562.

18. **Orth, G., F. Breitburd, M. Favre, and O. Croissant.** 1977. Papilloma viruses: possible role in human cancer, p. 1043–1068. *In* J. Watson (ed.), Origins of human cancer. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

19. **Orth, G., P. Jeanteur, and O. Croissant.** 1971. Evidence for and localization of vegetative viral DNA replication by autoradiographic detection of RNA-DNA hybrids in sections of tumors induced by Shope papilloma virus. Proc. Natl. Acad. Sci. U.S.A. 68:1876–1880.

20. **Osato, T., and Y. Ito.** 1968. Immunofluorescence studies of Shope papilloma virus in cottontail rabbit kidney

tissue cultures. Proc. Soc. Exp. Biol. Med. 128:1025–1029.

21. **Ozanne, B., P. A. Sharp, and J. Sambrook.** 1973. Transcription of simian virus 40 II. Hybridization of RNA extracted from different lines of transformed cells to the separated strands of simian virus 40 DNA. J. Virol. 12:90–98.

22. **Radloff, R., W. Bower, and J. Vinograd.** 1967. A dye-buoyant-density method for the detection and isolation of closed circular DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. 57:1514–1521.

23. **Rashad, A. L., and C. A. Evans.** 1967. A difference in sites of DNA synthesis in virus-induced (Shope) and in chemically induced epidermal tumors of rabbit skin. Cancer Res. 27:1639–1647.

24. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Bey.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 113:237–251.

25. **Rogers, S., J. G. Kidd, and P. Rous.** 1960. Relationships of the Shope papilloma virus to the cancers it determines in domestic rabbits. Acta Unio Int. Contra Cancrum 16:129–130.

26. **Sambrook, J., P. A. Sharp, and W. Keller.** 1972. Transcription of simian virus 40 I. Separation of the strands of SV₄₀ DNA and hybridization of the separated strands to RNA extracted from lytically infected and transformed cells. J. Mol. Biol. 70:57–71.

27. **Sharp, P. A., B. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose ethidium bromide electrophoresis. Biochemistry 12:3055–3063.

28. **Stevens, J. G.** 1978. Latent characteristics of selected herpesviruses. Adv. Cancer Res. 26:227–256.

29. **Wolf, H.** 1975. A procedure for simultaneous preparation of large amounts of DNA and RNA by the use of potassium iodide gradients. Anal. Biochem. 68:505–511.