JOURNAL OF VIROLOGY, Sept. 1981, p. 945-949
0022-538X/81/090945-05$02.00/0

Vol. 39, No. 3

NOTES

Unintegrated Viral DNA Sequences in a Hamster Tumor Induced by Bovine Papilloma Virus

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Received 26 January 1981/Accepted 8 May 1981

A fibrosarcoma was induced in a hamster by bovine papilloma virus type 2 (BPV2). The content of BPV2 DNA sequences was measured by DNA-DNA and cRNA-DNA hybridizations. The tumor contained approximately 300 BPV2 genome equivalents per cell. Southern blot hybridization indicated that the viral DNA was in free form, the entire genome most likely being present. In situ hybridization with BPV2 cRNA showed that multiple genome copies were present in each cell. Neither virus particles nor virus coat antigens could be detected in the tumor. A cell line was established from the fibrosarcoma, and the cells contained multiple copies of the BPV2 genome. The latter was in free form, and all of the DNA sequences appeared to be present in multiple copies and in all cells. An extensive search failed to reveal the presence of virus or viral antigens.

The bovine papilloma viruses (BPV) form a group of which five types have so far been characterized, BPV1 to 5 (5, 9, 12, 26; M. S. Campo, M. H. Moar, H. M. Laird, and W. F. H. Jarrett, Virology, in press). The classical cutaneous fibropapilloma virus (BPV2) commonly causes benign tumors of the head, neck, and thoracic skin. As its name implies, it is capable of transforming both mesenchymal and epithelial cells in its natural host (8, 9). In other species, such as the horse, mouse, and hamster, it induces fibromas and fibrosarcomas (3, 6, 21, 22). We describe experiments in which a tumor of fibroblasts was induced in a hamster by inoculation of BPV2. A cell line was established from this tumor (HT1). Both the tumor and the cell line have been examined for the presence of replicating virus, for virus antigens, for viral DNA sequences, and for the state in which these sequences occur. A spontaneous fibropapilloma containing BPV2 was removed from the neck of a cow, and a 10% suspension was made in phosphate-buffered saline. One milliliter of the suspension was injected subcutaneously into the back of a young hamster. After 2 years a large (5 by 3 cm) subcutaneous fibrous tissue was excised from the exact inoculation site. The tumor was actively mitotic and progressive but did not have metastases. The characteristics of similar hamster tumors have been described previously (6).

A piece of tumor was chopped into small fragments and suspended in growth medium (Eagle minimum essential medium plus 10% fetal bovine serum) at 37°C. The resulting monolayer became confluent after 10 days and was thereafter passaged by splitting it into three parts each week.

Individual isolates of purified BPV2 were prepared from tumors from individual spontaneous bovine cases and viral DNA was purified, both as described previously (5, 10; Campo et al., in press). Form I (supercoiled) BPV2 DNA was isolated by centrifuging the viral DNA in a CsCl-ethidium bromide gradient. This DNA was radioactively labeled by using either the method of nick translation (27) or *Escherichia coli* RNA polymerase (18). cRNA was synthesized by using [α-^{32}P]CTP or all four ribonucleoside triphosphates labeled with ^{3}H. The final specific activities of the labeled DNA or cRNA were estimated to be $\sim 4 \times 10^7$ and 1×10^8 cpm/μg (α-^{32}P) or 2×10^7 cpm/μg (^{3}H), respectively.

High-molecular-weight cellular DNA was extracted according to the method outlined by Parish and Kirby (25), with the inclusion of pancreatic RNase (Sigma Chemical Co.) enzymatic digestion and repeated phenol extraction. Restricted DNA was electrophoresed in slab agarose (Sigma) gels in E buffer (19), and the DNA fragments were transferred to nitrocellulose sheets (Schleicher & Schuell; 28) after de-

purination in situ (29). Blots were preincubated and thereafter hybridized to the α-^{32}P-labeled BPV DNA in 6× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate)-10× Denhardt solution (7) containing 10 μg of polyadenylic acid per ml, 10 μg of yeast tRNA per ml, 0.1% sodium dodecyl sulfate, and 50 μg of carrier calf testis DNA per ml (5; Campo et al., in press). The hybridized blots were dried and exposed to a presensitized X-ray film (Kodak X-Omat) at −70°C for several days (17).

Initially, filter disk hybridization was used to detect viral DNA in the hamster fibrosarcoma. DNA preparations were hybridized with BPV2 DNA or BPV2 cRNA. Table 1 shows that, on average, each cell of the original tumor contained approximately 300 BPV2 genome equivalents. A cell line, derived from the hamster tumor and designated HT1, contained multiple BPV2 genome equivalents as well: 25 at the sixth passage in tissue culture.

To analyze the state of the viral DNA in the tumor and in the cell line, we used restriction enzyme digestion of the DNA followed by hybridization of BPV2 DNA to Southern blot transfers (28). Hamster DNA was cleaved independently with the restriction enzymes *Eco*RI, *Hin*dIII, *Hin*dII, and *Hpa*II. The restriction sites for *Hin*dIII and *Hin*dII previously have been assigned coordinates on a circular genome map of BPV2 (9, 12; Campo et al., in press).

*Eco*RI does not cleave the genome, *Hin*dIII cleaves once, and *Hin*dII cleaves four times (Table 2). The *Hpa*II restriction sites have not been mapped on the BPV2 genome, but the molecular weights of the eight fragments produced are shown in Table 2.

In both the fibrosarcoma and the cell line (HT1) the BPV2 DNA hybridizes to DNA fragments which are identical in electrophoretic mobility to fragments produced by cleaving the original BPV2 DNA with these enzymes (Fig. 1). All three viral DNA forms exist: supercoiled molecules (form I), relaxed circular molecules (form II), and linear molecules (form III) (lanes d, h, and l). *Eco*RI does not cleave the viral DNA sequences (lanes c, g, and k). *Hin*dIII produces linear molecules with a molecular weight of 5.13×10^6, which is the molecular weight of form III BPV2 DNA (lanes b, f, and j). *Hin*dII produces four fragments corresponding to the fragments produced by cleaving BPV2 DNA (lanes a, e, and i), and *Hpa*II produces eight fragments with molecular weights identical to those of the fragments produced by cleaving BPV2 DNA (lanes m to o). (In this particular gel the lowest molecular weight fragment has run off the gel.) The hybridization intensity is higher in the tumor DNA than in the cell line (HT1) DNA, suggesting a lower number of viral genome equivalents in the cell line.

There is no hybridization to hamster kidney or hamster liver DNA, even after long exposure times. α-^{32}P-labeled BPV2 DNA was annealed to hamster tumor, HT1, or homologous BPV2 DNA, and the subsequently formed DNA-BPV2 DNA duplexes dissociated thermally in 0.1×

TABLE 1. *Filter disk hybridization of BPV2 DNA or cRNA to cellular and viral DNAs*

DNA[a]	cpm hybridized[b]		Estimated BPV2 genome equivalents[c]
	DNA	cRNA	
Hamster kidney	670	860	0
Hamster liver	890	960	0
Hamster tumor	48,000	58,000	300
Hamster cell line, passage 6	3,680	4,420	25
BPV2 (5 ng) + rat	19,000	23,500	150
BPV2 (2 ng) + rat	7,600	9,400	50
Rat	820	900	0
Human	800	890	0
Mouse	770	830	0

[a] 20 μg of DNA per filter.

[b] Average of three separate determinations. Conditions of hybridization: 6× SSC-10× Denhardt solution (7), 10 μg of polyadenylic acid per ml, 0.1% sodium dodecyl sulfate, 50 μg of calf testis DNA per ml, 10 μg of yeast tRNA per ml; 16 h at 70°C.

[c] Calculated based on 3×10^{12} (host) and 5×10^6 (BPV2) daltons (9).

TABLE 2. *Molecular weights of DNA fragments produced by restriction enzyme cleavage*[a]

Enzyme fraction	Mol wt ($\times 10^{-6}$)	Fractional length
*Hin*dII		
A	2.05	40.2
B	1.67	32.7
C	0.87	17.0
D	0.51	10.0
*Hpa*II		
A	2.3	46.2
B	0.89	17.8
C	0.48	9.6
D	0.40	8.0
E	0.34	6.8
F	0.26	5.2
G	0.17	3.4
H	0.14	2.8
*Hin*dIII	5.13	Form III (linear)

[a] The *Hin*dIII fragments of λ DNA were used as molecular-weight markers (15×10^6, 6.4×10^6, 4.3×10^6, 2.9×10^6, 1.6×10^6, and 1.4×10^6).

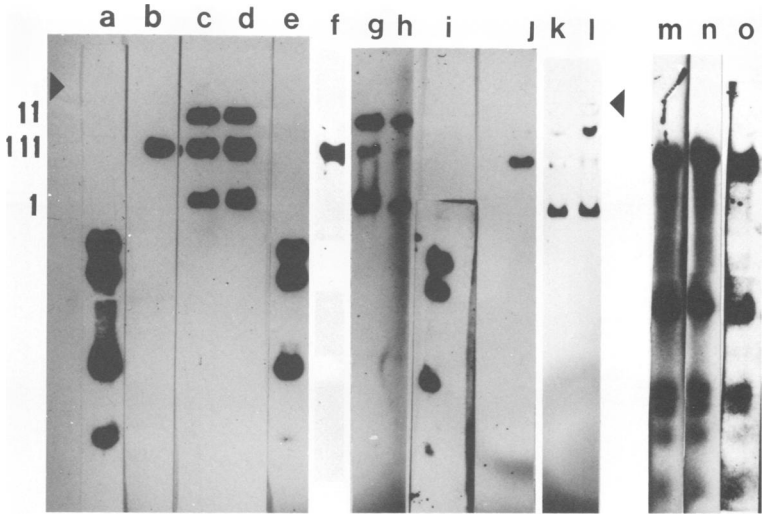

FIG. 1. *Autoradiograms of DNA restriction fragments hybridized to BPV2 DNA. (a–d, m) BPV2 DNA (5 ng); (e–h, n) hamster tumor DNA; (i–l, o) hamster HT1 DNA. (a, e, i) HindII restricted; (b, f, j) HindIII restricted; (c, g, k) EcoRI restricted; (d, h, l) unrestricted; (m–o) HpaII restricted. A 10-μg amount of DNA was loaded per sample, and the sample was electrophoresed on a 1% agarose gel. Exposure time was 3 days, except (a) and (i), for which exposure time was 9 days. The lowest-molecular-weight fragment in (e) and (i) is underrepresented in this particular gel; it is normally present in equimolar amounts compared with the other fragments. 1, 11, and 111 are forms I, II, and III of viral DNA, respectively. The origin of the gel is marked by arrowheads.*

SSC. The temperature at which 50% of a DNA-DNA duplex dissociates (T_m) is a measure of the sequence relatedness when heterologous and homologous duplexes are compared (4). The T_m's for hamster tumor, HT1, and BPV2-BPV2 DNA were identical (~62°C), indicating that the sequence homology between BPV2 DNA and the viral DNA in the hamster tumor or in the cell line is high.

To test whether a few cells in the tumor or cell line has disproportionate amounts of BPV2 DNA, we performed in situ hybridization with BPV2 cRNA. The BPV2 cRNA is complementary to >90% of the BPV2 genome, hybridizes to all eight *Hpa*II restriction enzyme fragments, and is therefore representative of the BPV2 genome (Moar, Campo, Jarrett, and Laird, manuscript in preparation). Figure 2a and b shows representative samples of the tumor and the HT1 cell line, in which no cells containing disproportionate amounts of BPV2 DNA could be detected in either case. In contrast, a large number of cells in the dysplastic epithelium of cattle fibropapillomas contained disproportionate amounts of viral DNA (Fig. 2c). Exposure times were increased to 2 months in the case of the hamster tumor and HT1 cell line, with no evidence of cells containing disproportionate amounts of viral DNA.

Electron microscopy was used to test for virus particles. Tumor tissue was fixed in paraformaldehyde-glutaraldehyde (11), postfixed in osmium tetroxide, and embedded in araldite. Thin sections were stained by uranyl acetate and lead citrate. Cultured cells were loosely pelleted and treated in the same way as tumor tissue.

The virus concentration and purification techniques described earlier (5, 10) were applied to the medium from cell cultures. Ten fractions ranging from 1.36 to 1.2 g/cm^3 were taken from CsCl density gradients. Identical fractions were taken from cultured cells disrupted by the techniques used for virus extraction (5, 10) and negatively stained by ammonium sulfate-phosphotungstic acid.

Five sections were cut from each of five blocks of tumor tissue, and 1,000 nuclear profiles were examined. No viral particles were found. A similar number of profiles of cultured cells from passage 6 were examined again without finding virus. Negatively stained preparations of disrupted cultured cells and medium at the same passage level were similarly negative for virions.

A large number of cultured cells of passage 5 were examined by using the peroxidase technique (Jarrett et al., manuscript in preparation), and in no case was any virion antigen detected. Negative results were also obtained by staining slides of the original hamster tumor. All tests were carried out in the presence of slides of BPV2-induced skin fibropapillomas in which virus antigen could be easily detected in the gran-

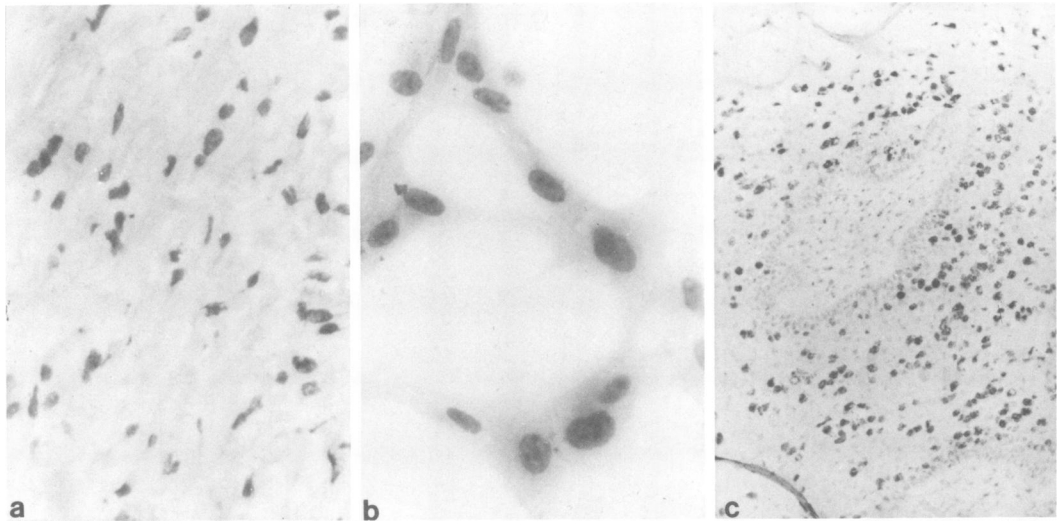

FIG. 2. *In situ hybridization of ^{3}H-labeled BPV2 cRNA to (a) hamster fibrosarcoma, (b) HT1 cell line, and (c) bovine cutaneous neck fibropapilloma. Exposure time was 3 weeks. Cells were fixed in methanol-acetic acid (3:1), the DNA was denatured with 0.05 N NaOH, and hybridization was performed in 3× SSC at 60°C for 20 h with 5 μg of cRNA per ml (20). Over 1,000 cells were screened.*

ular layer of the epithelium.

BPV induces fibromas, fibrosarcomas, and sarcomas in heterologous (3, 6, 21, 22) hosts, and BPV DNA sequences have been detected in these tumors (14, 16). Naturally occurring equine sarcoids also possess BPV DNA sequences and may therefore be induced by BPV also (15). Hamster fibrosarcomas have previously been shown to contain BPV DNA, but neither the inducing virus nor the state of the virus DNA in the tumors was characterized (14, 16). Here, we have induced a hamster fibrosarcoma with BPV2, the virus associated with classical cutaneous fibropapillomatosis in cattle (9; Campo et al., in press). The tumor contains multiple copies of the BPV2 genome, the complete viral genome appears to be present, and, most interesting, the viral DNA occurs in an unintegrated free form.

In situ hybridization with BPV2 cRNA, however, has failed to reveal cells within the tumor which contain disproportionate amounts of BPV2 DNA. It is unlikely, therefore, that the multiple copies of BPV2 DNA found in the tumor are due to virus DNA replication within a few cells. We have also failed to detect any virus particles or virus antigens within the tumor cells. The lack of virus particles is in agreement with the work of others analyzing other cases of hamster fibrosarcoma induced by BPV (3, 14, 16). Extracts of hamster fibrosarcomas induced by BPV also fail to transform fetal bovine conjunctiva, in contrast to extracts known to contain BPV (2). As with Shope papilloma virus (24) and human papilloma virus (23), the only cell type in which papilloma virus particles have thus far been detected is epithelial (Moar et al., in preparation). The failure to detect virus particles and the ability to detect multiple free viral DNA copies in the hamster fibrosarcoma suggest that these cells carry papilloma virus DNA in an episomal state and that the virus cycle is restricted.

We have, in addition, established a cell line (HT1) derived from the hamster fibrosarcoma. It contains multiple copies of the BPV2 genome, the complete viral genome appears to be present, and the viral DNA is in a free form. As in the original tumor, no antigens or virus particles have been detected, and in situ hybridization tests have failed to detect disproportionate viral DNA synthesis. We are currently analyzing a variety of clonal cell lines derived from HT1 to determine whether a limited amount of viral DNA integration occurs which remains below the detection levels of the present experiments or may be masked by the high amounts of free viral DNA.

Recently, there have been other reports of unintegrated bovine papilloma virus DNA in tumor cells and transformed cells (1, 13; Moar et al., submitted for publication). Such apparent lack of integration of viral DNA into the host cell genome raises the question of alternate mechanisms by which tumor viruses transform cells in vitro and in vivo.

This work was carried out with the aid of grants from the Cancer Research Campaign (grant 916/800/2), the Agricul-

tural Research Council (grant AG 17/112), and the Public Health Service National Institutes of Health (grant N01 CP 81022). M.S.C. is a recipient of a Cancer Research Campaign Career Development Award.

We gratefully acknowledge the excellent assistance of Ellen Leighton, Celia Burke, Carole Mckay, and B. W. O'Neil.

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