Integrated Shope Virus DNA is Present and Transcribed in the Transplantable Rabbit Tumour Vx-7

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SUMMARY

To facilitate a molecular analysis of Shope papilloma virus-induced neoplastic cells, we have established a cell line from Vx-7, a transplantable tumour originally induced by the Shope virus. Single phase molecular hybridization and Southern transfer methods were employed to assess copy number and physical state of the DNA, and the extent of transcription. Both tumour and cell line were found to contain multiple copies of the virus genome and these were all integrated into the host cell DNA. Transcripts corresponding to a complexity of approx. 1% of the virus genome were detected at low abundance. These results are discussed relative to our earlier findings with tumours induced directly by virus, and to requirements for maintenance of the Vx-7 tumour over the 30 years that it has been in existence.

INTRODUCTION

In previous studies, we showed that cells comprising benign and malignant neoplasms induced on domestic rabbits by the Shope virus contained multiple copies of the virus genome, and that RNA transcripts of limited complexity were present at low abundance in these tumours (Stevens & Wettstein, 1979; Wettstein & Stevens, 1980). In addition, the DNA was found to be present as both integrated sequences and as plasmids of varying sizes (F. O. Wettstein & J. G. Stevens, unpublished results). To further define molecular events related to the neoplastic transformation initiated by this virus, a cell culture system would obviously be very useful. For this reason, we spent considerable effort attempting to establish continuous lines of cells from both papillomas and primary or metastatic carcinomas, but were unsuccessful.

As an alternative, we have established a cell line from the Vx-7 carcinoma, a transplantable Shope virus-induced carcinoma initiated some 30 years ago in the laboratory of Dr P. Rous (Rogers et al., 1960). In this communication, we show that multiple copies of virus DNA are present in an integrated state in both the tumour cells and in a tumour-derived cell line. In addition, preliminary evidence indicates that, as in the tumours already studied, the virus genome is transcribed to a limited extent in both the tumour and cell line.

METHODS

The Vx-7 tumour. The initial source of the Vx-7 tumour employed, its anatomical characteristics, and the methods used for transmission to, and propagation in rabbit muscle have been described by Evans et al. (1962). This tumour, maintained in continuous rabbit passage by one of us (Y.I.) since 1962, was sent to Los Angeles in 1978, and has been kept as
frozen stocks in liquid N\textsubscript{2} between passages to rabbits. When 0.5 ml of a 20\% (v/v) suspension of packed tumour cells is inoculated intramuscularly, cystic tumours about 2 inch in diam. develop in 4 to 6 weeks. The neoplastic epithelial lining of the cyst is stripped and is used as a source of tumour cells.

**Establishment of the Vx-7 cell line.** Initial attempts to establish cultures by direct seeding of cells separated by various standard methods, and then maintaining them in several media formulations did not result in establishment of an immortalized culture. The principal difficulty in all instances was initial overgrowth with, and displacement of, tumour cells by contaminating fibroblasts. To select for the neoplastic cells in these tumours, cells were inoculated (0-1 ml prepared as above) subcutaneously into outbred athymic nude mice (Harland Laboratories, Indianapolis, Ind., U.S.A.). In 3 to 4 weeks, after tumours 0.5 inch in diam. had developed, the mice were killed, tumours removed and prepared for culture by the following method. One mm\textsuperscript{3} pieces of minced tumour tissue were incubated overnight at 37 °C in Eagle’s minimal essential medium (MEM) plus non-essential amino acids (NEAA), 20\% foetal calf serum and collagenase (1 mg/ml). The clusters of neoplastic cells obtained were allowed to settle by gravity and these were washed several times with MEM. MEM with 20\% foetal calf serum and NEAA was re-added (1% suspension of cells), the suspension was seeded at 5 ml/25 cm\textsuperscript{2} plastic flask and incubated at 37 °C. When the cells became about 75\% confluent (2 weeks), they were split 1:2 and repassaged. After some 10 such passages, they were changed to MEM-NEAA plus 10\% adult bovine serum, and have been maintained in continuous passage (approx. 70 additional passages at 5- to 6-day intervals) since that time. A karyotype analysis kindly performed by Dr H. Müller of the Division of Medical Genetics, Department of Medicine at this institution confirmed the rabbit origin of these cells.

**Isolation and purification of nucleic acids from tumour and cell cultures.** For use in liquid hybridizations, tumours snap-frozen in liquid nitrogen were ground, digested and extracted as described previously (Stevens & Wettstein, 1979). To separate RNA from DNA, the sample in a CsCl solution of density 1.69 g/ml was layered on 1 ml of a solution of density 1.82 g/ml and centrifuged at 30000 rev/min for 24 h at 25 °C (SW50.1 rotor, Beckman). The RNA pellet was redissolved in buffer (0.1 M-NaCl, 0.01 M-tris pH 7.4, 0.001 M-EDTA), precipitated with ethanol and stored at -20 °C. The DNA band was collected, dialysed against the same buffer, and also precipitated with ethanol. Before use, the purified RNA and DNA were pelleted, dried and redissolved in either formamide (RNA) or water (DNA). Samples of RNA to be used as controls were hydrolysed with NaOH as described previously (Wettstein & Stevens, 1981). High molecular weight DNA for analysis by the Southern blotting technique was extracted from ground tumour tissue by the method of Blin & Stafford (1976) except that 5\% SDS was used during proteinase K digestion and extraction was with phenol–chloroform–isoamyl alcohol (75:24:1) equilibrated to pH 8. To prepare cell cultures for digestion, monolayers were rinsed in cold phosphate-buffered saline (PBS), and then scraped and centrifuged at 400 g for 15 min. The cell pellets were then treated in a manner identical to ground tumour tissue. Fifteen flasks (75 cm\textsuperscript{2}) of cells usually yielded from 5 to 10 mg of RNA or DNA.

**Hybridizations.** Probes for single phase liquid hybridization consisted of Shope DNA cloned in pBR322, which was excised from the recombinant molecule, purified on sucrose gradients (Wettstein & Stevens, 1980) and labelled with \textsuperscript{3}Hthymidine by nick translation (Rigby \textit{et al.}, 1977) to specific activities of 4.1 \times 10\textsuperscript{6} to 10.5 \times 10\textsuperscript{6} ct/min/\mu g. Intramolecular double strands were removed as described previously (Stevens & Wettstein 1979). DNA–DNA reannealing was in aqueous phosphate buffer (Stevens & Wettstein, 1979) and RNA–DNA hybridization was in 70\% formamide (Wettstein & Stevens, 1981).

**Analysis of tumour DNA by Southern blotting.** Gel electrophoresis of DNA was in 0.7\% agarose containing 0.5 µg/ml ethidium bromide (Sharp \textit{et al.}, 1973) at 1.7 V/cm for 16 h.
Integrated Shope virus DNA

Fig. 1. Kinetics of $^3$H probe reannealing to DNA isolated from Vx-7 tumours and Vx-7 tissue cultures. DNA was isolated from Vx-7 tumours or Vx-7 tissue cultures sonicated and denatured. Reannealing of DNA (2.5 mg/ml) to $^3$H-labelled Shope DNA probe (40000 ct/min/ml, 4.1 x 10$^6$ ct/min/μg) in aqueous phosphate buffer and analysis of the extent of reannealing were performed as described previously (Stevens & Wettstein, 1979). Each point represents the average of duplicate analysis. ■, Vx-7 tumour DNA; ●, Vx-7 tissue culture DNA; ▲, calf thymus DNA.

RESULTS

Quantitative determination of virus DNA present in the Vx-7 cells

Since the major reason for deriving a cell line was to study in detail the expression of the virus genome, it was obviously of initial importance to establish that the genome was indeed present in these cells. To detect and quantify virus-specific DNA, single phase molecular hybridization was employed, and DNA from the cell line as well as from the tumour was analysed. As can be seen in Fig. 1, DNA from both tumour and cell line accelerated the rate of reassociation of the probe, indicating that virus genetic information was present. The data in this figure also suggest that the entire genetic complement of the virus is present. Thus, the slope of the curve and the final extent of reannealing for both probe and tumour cell DNA are
Fig. 2. Electrophoretic analysis of tumour DNA. High mol. wt. DNA was isolated from Vx-7 tumours or Vx-7 tissue cultures and 2.5 µg were applied per track of a 0.5 cm-thick 0.7% agarose gel as described in Methods. Tracks 1 and 2 were stained with ethidium bromide and tracks 3 and 4 represent autoradiograms of Southern blots prepared as described in Methods. Tracks 1 and 3, Vx-7 tissue culture DNA; tracks 2 and 4, Vx-7 tumour DNA. The positions of HindIII fragments (23, 9.5, 6.4 and 4.2 kilobase pairs) of phage lambda DNA are indicated; S shows the expected position of supercoiled Shope DNA. The relative intensity of the bands suggests that tissue culture DNA contains more virus-specific DNA than does the tumour DNA, and this would be in contradiction to the results presented in Fig. 1. Two factors contribute to this apparent discrepancy, and the first relates to the quality of the DNA preparations. DNA isolated from tumours is always somewhat degraded and a significant portion remains at the top of sucrose gradients. In the gel shown here, this DNA is barely detectable, and most of it moves beyond that segment of the gel illustrated. However, there is an indirect indication of high mol. wt. DNA loss in this figure since the stained DNA band from tumours is somewhat less intense than the band formed by cell culture DNA. Presumably, this degraded DNA (oligonucleotides) would be detectable in single phase hybridization experiments. The second factor concerns sampling variations. Since it is probable that the number of malignant versus normal cells varies between tissue specimens, an apparent variation in the relative content of virus DNA would result.
equivalent to the results derived when the probe was allowed to anneal to itself. Using \( C_{m/2} \) values derived from these curves, with standards and methods detailed earlier (Stevens & Wettstein, 1979), the virus genome equivalents per diploid cell amount of DNA were calculated. A value of 28 was obtained for the tumour, and 9 for the cell line derived from it. Although these results will be discussed in further detail later, the presence of virus genetic information in multiple copies in these tumours over decades suggests that persistence of the virus genetic information is critical for maintenance of the neoplastic state.

**Physical state of virus DNA in the Vx-7 cell**

Recent studies of bovine and rabbit papilloma virus DNAs (see Law et al., 1981; F. O. Wettstein & J. G. Stevens, unpublished results) have shown that the virus genomes generally exist in a non-integrated state. It was of interest to determine whether such a state persisted in the transplantable tumour. In order to distinguish between integrated and non-integrated virus DNA, analyses by agarose gel electrophoresis and Southern blotting were employed. The data in Fig. 2 indicate that virus-specific sequences are present in a diffuse band which co-migrates with cellular DNA, thus suggesting that these sequences are integrated. To provide more direct evidence for virus DNA integration, Vx-7 tissue culture DNA was digested with the restriction enzyme EcoRI. This enzyme cuts DNA of the Washington B strain of Shope papilloma virus once (Stevens & Wettstein, 1979). A Southern blot of digests hybridized with the virus genomic probe is shown in Fig. 3 (a) (track 1), and one very strong band with the mobility of linear unit length virus DNA, plus five or six weak bands, which may be integration pieces, are present. An identical pattern was obtained when DNA initiated from Vx-7 tumour was analysed (data not shown).

Since the two slowest moving weak bands had mobilities of linear dimers and trimers of virus DNA, their nature was further investigated. In these studies, the Southern blots of EcoRI-digested DNA were hybridized with four different subgenomic probes representing the whole virus genome. Here, virus-specific bands which do not hybridize to all four probes would represent integration bands, while those hybridizing to all probes would represent complete virus genomes in the form of monomers, dimers and trimers or integration bands containing a large segment of DNA. The results of these analyses are presented in Fig. 3 (tracks 2 to 5). As expected, the major band hybridizing with whole genomic probe and which had the mobility of linear virus DNA represented again the strongest band and it did hybridize with all four probes. The band with the mobility of a linear dimer also hybridized to all four probes, and it may thus represent a dimer resulting from incomplete digestion or it could be an integration band in which the virus segment contains a large fraction of the genome. The other three bands did not hybridize to all of the subgenomic probes. This identifies them as integration bands. The fastest moving band, which appeared to be a double band, hybridized strongly to two probes which do not represent contiguous segments of the virus genome. This suggests that the double band indeed represents two different virus–cellular junction pieces. Taken together, these data indicate that at least five and possibly six integration bands are present, and no evidence for non-integrated DNA was found.

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Fig. 3. Analysis of EcoRI-digested Vx-7 tumour DNA. (a) High mol. wt. DNA was isolated from Vx-7 tissue cultures as described in Fig. 2 and Methods. DNA was digested with 20 units EcoRI restriction endonuclease (New England Biolabs) per 5 \( \mu \)g DNA for 2 h at 37 °C under conditions recommended by the supplier. A 5 \( \mu \)g amount of digested DNA was applied per track of a 1 cm-thick 0.7% agarose gel, electrophoresed, blotted and hybridized with different probes as described in Methods. The probes for the different tracks were as follows: track 1, whole genomic probe; track 2, BgII fragment 1; track 3 BgII fragment 2; track 4, BgII fragment 3a; track 5, BgII fragment 3b. (b) Arrangement of BgII fragments in Shope virus DNA (Washington B strain) linearized with EcoRI. The arrangement of the BgII fragments was derived from partial digests.
Fig. 4. Melting curves of $^3$H-labelled Shope DNA probe reannealed in the presence of calf thymus DNA or reannealed to Vx-7 cell culture DNA. $^3$H-labelled Shope DNA probe (15,000 ct/min/ml, $4.1 \times 10^6$ ct/min/µg) was reannealed in the presence of 2.5 mg/ml sonicated denatured calf thymus DNA or Vx-7 tissue culture DNA for 5 days at 65 °C in aqueous phosphate buffer as described in Methods. Fifty µl amounts of the reannealing mixture were sealed in capillaries and incubated for 10 min at the indicated temperatures. Duplicate samples were chilled, diluted and analysed. In the reannealing mixtures of probe with Vx-7 tissue culture DNA, 70% of the virus-specific sequences represented Vx-7 DNA and 30% were probe DNA. ○, Probe reannealed in the presence of calf thymus DNA; □, probe reannealed in the presence of Vx-7 DNA.

Fig. 5. Hybridization in 70% formamide of RNA isolated from Vx-7 tumours and Vx-7 cell cultures to $^3$H-labelled Shope DNA probe. RNA was isolated from Vx-7 tumours of Vx-7 tissue cultures as described in Methods. Hybridization of RNA (5 mg/ml) in 70% formamide to $^3$H-labelled Shope DNA probe (4000 ct/min/ml, $10.5 \times 10^6$ ct/min/µg) and analysis of the hybridization mixture were performed as described previously (Wettstein & Stevens, 1980). All time points represent the average of duplicate analysis, and radiolabelled fractions were counted for 40 min each. (a) Vx-7 tumour RNA; (b) Vx-7 tissue culture RNA. ○, Hybridization to RNA; ▲, hybridization to base-treated RNA; ■, hybridization to tRNA.

Melting profile of Vx-7 probe DNA hybrids

The capacity of DNA from the Washington B strain of Shope papilloma virus to serve as a probe for virus sequences in Vx-7 DNA suggested that considerable homology existed between the two sources of the DNA sequences. To quantify the extent of this homology, the thermal stability of double-stranded probe DNA was compared to that of probe Vx-7 DNA hybrids. The results are presented as melting curves in Fig. 4. There is no indication of a significant difference between the stability of the two DNA duplexes studied, and from an inspection of these curves, we estimate that a 2 °C difference in the melting point would have been detectable. Extrapolating from studies involving SV40 and BK virus DNA hybrids (Yong & Wu, 1979), these results indicate that the degree of nucleotide mismatch between the probe and Vx-7 DNA could not be greater than 3%.

Transcription of the virus genome in Vx-7 cells

A possible reason for the retention of virus DNA in Vx-7 tumours and the Vx-7 cell line is that some sequences may be transcribed and their translation products could be crucial to maintenance of the neoplastic state of the cells. In previous experiments with Shope virus-induced papillomas and carcinomas (Wettstein & Stevens, 1980), we have been able to detect virus transcripts by single phase liquid molecular hybridization in formamide. The studies here were performed similarly, and the results of a typical hybridization experiment are presented in Fig. 5. They indicate that limited and equivalent transcription of the virus genome occurs in both the Vx-7 tumour and the Vx-7 cell line. DNA extracted from the Vx-7 tumour was used to drive the reaction in Fig. 5 (a), and it can be seen that virus-specific RNA.
Integrated Shope virus DNA was detected at a $R_{av}$ of about $5 \times 10^2$, and at an approx. $R_{av}$ of $5 \times 10^3$ (after subtracting radioactivity contributed by the base-treated control), some 10% of the virus DNA probe was found to be hybridized. The increased hybridization to base-treated control over the tRNA background control at high $R_{av}$ values in this experiment is due to DNA contamination of the RNA preparation. Results with RNA from the cell line are presented in Fig. 5(b). Again, about 10% of the DNA was driven into a hybrid, with reaction kinetics similar to those of Fig. 5(a). If a correction is made for the incomplete reannealing of the probe, which was about 90% (Fig. 1), then some 11% of the virus genome is transcribed in both tumours and tissue cultures.

**DISCUSSION**

In this communication, we have shown that in neoplastic cells populating the Vx-7 carcinoma, multiple genome equivalents of Shope virus DNA are maintained. These virus sequences are integrated into the cellular genome. In addition, the virus genetic information in these cells is transcribed into RNA homologous to approx. 11% of the virus genome or, if it is assumed that transcription is asymmetric, 22% of the available genetic information.

Several aspects of these results deserve comment. First, there was some initial concern over the Washington B strain of virus as a probe for virus DNA in the Vx-7 tumour. This was an important potential difficulty since it has been shown that strains with significantly different antigenic and nucleotide compositions exist in both human (Gissmann & zur Hausen, 1976; Orth et al., 1977, 1978; Pfister & Gissmann, 1978; Heilman et al., 1980) and bovine (Lancaster & Olson, 1978; Lancaster, 1979; Law et al., 1979; Pfister et al., 1979) papilloma viruses. In addition, the opportunity for selection, over time, of unique virus sequences in Vx-7 was a definite possibility. However, our results indicate that the probe is suitable since the shape of annealing curves between Vx-7 virus DNA and Washington B DNA were indistinguishable from those derived for the annealing of Washington B DNA to itself. In addition, all restriction endonuclease fragments of the Washington B DNA studied hybridize to their counterparts in Vx-7 DNA, and there was no detectable difference in thermal stability of Vx-7—Washington B virus DNA hybrids and duplexes of Washington B DNA.

The second aspect relates to the molecular nature of the Vx-7 virus DNA. The copy number and physical state of this DNA differs from our earlier findings in tumours induced by virus directly. The number of virus genome equivalents per cell in those tumours was five to ten times greater than that detected in Vx-7, and the virus DNA was present in plasmids of varying sizes. Here, no distinct bands characteristic of plasmids could be detected in Southern blots of undigested DNA, and all virus-specific sequences co-migrated with cellular DNA. This was preliminary evidence that these sequences were integrated, and additional support for this was obtained from the analyses of EcoRI restriction digests. In these digests, at least four, and possibly six, integration bands were detected. This result indicates that at least two, and possibly three, different sites of virus DNA integration are present. This number is smaller by a factor of 3 than the number of virus genomes detected per diploid cell DNA equivalent and, since these cells are polyploid, the difference between number of integration sites and number of integrated genomes per cell is even greater. These results, coupled with the finding that unit length virus DNA is the major product of EcoRI digestion, suggest that virus genomes are integrated as head to tail tandem repeats.

The third point concerns extent of transcription in Vx-7 tumours and the cell culture. In spite of the difference in abundance and physical state of the DNA in these cells and tumours induced by virus directly, the complexity and abundance of transcripts detected in Vx-7 did not differ significantly from those found in the previous studies. It therefore appears that fewer copies of virus genetic information encoding what may well be equivalent transcripts are maintained in a quite different physical state in Vx-7, and the significance of this
difference is not clear. However, since virus transcripts are present, it can be argued that they serve the crucial function of encoding proteins necessary for maintenance of the neoplastic state. Possibly, episomal virus DNA would be lost, and only integrated sequences could survive the 30 year period in which this tumour has been in existence.

Finally, it is of obvious importance to obtain precise information concerning the transcripts and their presumed translation products, and these aspects are being vigorously pursued.

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REFERENCES


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