Two Colinear and Spliced Viral Transcripts Are Present in Non-Virus-Producing Benign and Malignant Neoplasms Induced by the Shope (Rabbit) Papilloma Virus

MOHAMMAD NASSERI, FELIX O. WETTSTEIN,* AND JACK G. STEVENS

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

Received 15 March 1982/Accepted 17 June 1982

The nature of Shope virus-specific RNA was investigated in non-virus-producing Shope (rabbit) papilloma virus-induced benign and malignant domestic rabbit tumors and in a cell line derived from the VX-7 transplantable carcinoma. RNA transfer (Northern) blot analysis of polyadenylated RNA isolated from whole cell extracts of all three sources was resolved into two major bands of 1.3 and 2.0 kilobases. Two additional minor bands of 3.5 and 4.8 kilobases could be seen in some analyses. RNA of the VX-7 cell line was further separated into nuclear, cytoplasmic, and polysomal fractions. The cytoplasmic fraction only contained the 1.3- and 2.0-kilobase species, and virus-specific RNA was found to be associated with polysomes. The two major transcripts present in VX-7 cells were mapped by hybridization of RNA transfer blots with subgenomic probes, and the results indicated that both transcripts are spliced and are most likely colinear. Our results are consistent with the suggestion that the two viral transcripts are necessary for induction and maintenance of the neoplasms.

Shope (rabbit) papilloma virus induces papillomas in domestic rabbits, and carcinomas usually develop at the same site several months later. Although these neoplasms are non-virus producing, both types contain from 10 to over 100 viral gene copies per cell (15), and most of the viral DNA present is extrachromosomal. In papillomas, the free viral DNA consists mainly of unit-sized circles, whereas in carcinomas, oligomeric large circles predominate. In some tumors (of both types), up to 25% of the viral DNA is integrated (19). Viral transcripts complementary to between 6 and 12% of viral DNA (100% would represent RNA hybridization to both DNA strands) were detected in low quantities in all tumors (18).

The two-stage induction of a malignant tumor is one of the unique features of the Shope papilloma-carcinoma system, and an important question is whether tumor progression is paralleled by changes in viral gene expression (which may be responsible for the tumor progression). However, since it has not been possible so far to identify virus-specific antigens by immunological techniques in non-virus-producing tumors induced by any papilloma virus (4, 8, 9, 14), a direct approach for answering this question is not yet available. In an indirect approach, we have characterized, by RNA transfer (Northern) blotting and mapping, viral transcripts in benign

and malignant tumors induced by the Shope (rabbit) papilloma virus. Two major spliced colinear transcripts of 1.3 and 2.0 kilobases (kb) are present in both types of tumors, as well as in a cell line derived from the transplantable Shope virus-induced VX-7 carcinoma. Our data further suggest that there is a difference in the relative abundances of the two transcripts between benign and malignant tumors.

MATERIALS AND METHODS

Animals, viruses, and VX-7 cell lines. The sources of animals and virus and the mode of infection were described previously (14). The cell line derived from the transplantable VX-7 tumor (7a) was grown in Eagle minimal essential medium plus nonessential amino acids and 10% bovine serum at 37°C in 5% CO₂. Cells were split 1 to 2 every 3 or 4 days.

Isolation of RNA. Shope virus induced-tumors were ground frozen into a powder, and nucleic acids were isolated by the guanidinium hydrochloride extraction procedure (3). The guanidinium hydrochloride extract was then subjected to brief sonication to prevent coprecipitation of DNA with RNA. Guanidinium hydrochloride extraction was used in these investigations since standard hot phenol extraction of frozen tumor powders yielded only degraded RNA, and this degradation could not be reduced adequately when various RNase inhibitors (heparin, vanadyl ribonucleoside complexes [2], and diethyl pyrocarbonate) were included. Polyadenylated [poly(A)⁺] RNA was selected

by two successive passages over oligodeoxythymidylate [oligo (dT)] cellulose columns (1).

For the isolation of RNA from tissue cultures, cells were harvested by trypsinization followed by a wash in trypsin inhibitor (6). For cell fractionation, the cellular pellet was suspended in 0.1 M KCl–0.01 M Tris-hydrochloride (pH 7.6)–0.005 M $MgCl_2$ (TKM buffer) containing 0.01 M vanadyl ribonucleoside complexes (2), and the cells were lysed by the addition of 0.1 volume of 5% deoxycholate–10% Nonidet P-40 and brief homogenization in a tight-fitting Dounce glass homogenizer. The nuclei were pelleted at 2,500 rpm for 10 min at 0°C. Polysomes and free ribonucleoprotein complexes were precipitated from the cytoplasmic extract with $MgCl_2$ (10). To obtain polysomes separate from ribonucleoprotein complexes, we layered a cytoplasmic extract over 2.4 ml of 0.5 M sucrose made in TKM buffer containing 0.01 M vanadyl ribonucleoside complexes. The polysomes were pelleted by centrifugation at 50,000 rpm for 1 h at 4°C in an SW50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.) Poly(A)⁺ RNA was isolated from whole cells or subcellular fractions by guanidinium hydrochloride extraction and oligo(dT)-cellulose selection as described above for tumors.

Mapping of *MspI* and *BglII* fragments. Two methods were used to map the restriction fragments. In the first approach, Southern blots (13) were prepared from *MspI*-digested Shope DNA isolated from a cottontail rabbit papilloma and hybridized with probes consisting of nick-translated (11) electrophoretically separated restriction fragments obtained by *BglII*-*EcoRI* double digestion of the Shope-pBR322 recombinant plasmid (17). In addition, Southern blots were also prepared from *MspI* digests of purified *BglII*-*EcoRI* fragments and hybridized with full genomic probe. The arrangement of the *MspI* fragments within *BglII* fragment I was deduced from partial digests of the fragment with *MspI*. The second approach, which permitted the mapping of several additional small fragments that could not be detected by Southern blotting, involved the mapping procedure of Smith and Birnstiel (12). Shope DNA excised from the recombinant plasmid was purified by sucrose gradient centrifugation (17). The linear Shope DNA was end labeled with ³²P by T4 polynucleotide kinase after removal of terminal phosphates with alkaline phosphatase. The end-labeled DNA was then cut into two fragments by *SalI* digestion (19), and the fragments were separated by agarose gel electrophoresis. The fragments were electroeluted, extracted with phenol, and recovered by ethanol precipitation. The reisolated fragments were digested to different extents with *MspI* by using different amounts of enzyme. The digests were then analyzed by electrophoresis on 1 and 2% agarose gels followed by autoradiography.

RNA analysis by RNA transfer (Northern) blot hybridization. Poly A⁺ RNA was glyoxalated (7) and electrophoresed on 1.1% agarose gels in 0.01 M sodium phosphate buffer (pH 7) at 2 V/cm for 6 h. The RNA was transferred to cellulose nitrate filters and hybridized (16) with nick-translated (11) full genomic or subgenomic probe (15). The ³²P-labeled TTP or dCTP used in the nick translation had specific activities of 2,000 to 3,000 Ci/mmol and were purchased from Amersham Corp., Arlington Heights, Ill., or from New England Nuclear Corp., Boston, Mass. The

specific activity of full genomic probes was 10⁹ cpm/μg. After hybridization, the filters were washed five times at room temperature in 2× SSC (1× SSC equals 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate, followed by four washes at 50°C in 0.1× SSC–0.1% sodium dodecyl sulfate; autoradiography was at –70°C with amplifying screens (DuPont Cronex Lightning Plus).

RESULTS

In our previous investigation (18), we showed by single-phase liquid hybridization that non-virus-producing papillomas and carcinomas contain viral transcripts of similar complexity and low abundance. To probe in more detail into the nature of the viral transcripts present in the two types of tumors, analysis by RNA transfer (15) (Northern) blotting appeared to be the method of choice. This method of analysis requires, however, that undegraded RNA can be isolated. Our preliminary investigation indicated that standard hot phenol extraction did not yield RNA of a quality adequate for RNA transfer blotting, and other RNA isolation procedures were evaluated. The method yielding the best results was a modification of the guanidinium hydrochloride extraction (3) described above.

The blot analyses of oligo(dT)-cellulose-selected RNA isolated from Shope virus induced-tumors are shown in Fig. 1. Track 1 represents the analysis of rRNA, the high-salt eluate from oligo(dT)-cellulose. The well-defined bands indicate that the integrity of RNA molecules the size of rRNA is not impaired by brief sonication. Virus-specific RNA from a papilloma is shown in tracks 2 and 3, and that isolated from a carcinoma is shown in tracks 4 and 5. Both types of tumor contain two major bands of 1.3 and 2.0 kb. The relative intensity of the two bands is reversed between papilloma and carcinoma. However, the quantitation of such differences is relatively inaccurate, since minor degradation (as shown in the carcinoma blot) or variations in the efficiency of transfer could measurably affect the result. The RNA analyses thus suggest that viral transcripts in papillomas and carcinomas are similar.

The presence of at least two viral transcripts in these tumors predicts the existence of two virus-specific proteins. However, virus-specific antigens have not yet been identified in any of these tumors. Therefore, it appeared to be important to determine if viral transcripts could be detected in the cytoplasm and found associated with polysomes. Since fractionation of carcinomas and papillomas into nuclear and cytoplasmic fractions is not technically feasible (because of the tough nature of the tumors), these analyses were carried out with a cell line derived from a transplantable Shope virus-induced domestic rabbit carcinoma. The tumor and the cell line

FIG. 1. RNA transfer (Northern) blot analysis of virus-specific transcripts in Shope (rabbit) papilloma-induced benign and malignant tumors. Total cellular RNA was isolated from tumors, and poly(A)⁺ RNA was selected and analyzed by RNA transfer (Northern) blotting and hybridization with whole genomic probe. Track 1, rRNA (5 μ g) eluted from oligo(dT)-cellulose columns with high salt was electrophoresed and stained with ethidium bromide; tracks 2 and 3, poly(A)⁺ RNA isolated from a papilloma (both tracks contained 19 μ g of poly(A)⁺ RNA); tracks 4 and 5, poly(A)⁺ RNA isolated from a carcinoma (track 4, 20 μ g of poly(A)⁺ RNA; track 5, 10 μ g of poly(A)⁺ RNA). The bars indicate the position of 18S and 28S rRNA. The origin of the gels is not shown; the RNA in tracks 4 and 5 was run farther than that in tracks 1 through 3.

contain 10 to 20 viral gene copies per diploid cell DNA equivalent, and all of it is integrated. Viral transcripts in low abundance and homologous to 11% of the DNA are also present (7a), and this amount of RNA is similar to that found previously in non-virus-producing domestic rabbit tumors directly induced by the virus (18). As in the directly induced tumors, the RNA transfer blots of poly A⁺ RNA isolated from total cellular RNA show two major bands of 1.3 and 2.0 kb (Fig. 2A, track 1), and both were also present in poly(A)⁺ RNA isolated from the cytoplasmic fraction (Fig. 2A, track 2). A smear visible in the autoradiogram of the cytoplasmic RNA suggests that some degradation does occur during cell fractionation. The cytoplasmic fraction was further separated into polysomes and a postpolysomal supernatant, and sucrose gradient analysis showed that part of the free 80S ribosomes and most of the native 60S and 40S ribosomal sub-

units remained in the supernatant fraction (data not shown). RNA was extracted from the polysomal pellet directly and from the postpolysomal supernatant after addition of HeLa cell polysomes as carrier. The analysis of the poly(A)⁺ RNA (Fig. 2B) shows that viral sequences are detectable in the polysomal (track 2) fraction but not in the postpolysomal (track 3) fraction. The virus-specific RNA in the polysome fraction is not resolved into distinct bands. This is most likely the result of some degradation during fractionation, which did occur despite the presence of vanadyl ribonucleoside complexes (2).

FIG. 2. RNA transfer (Northern) blot analysis of whole cell and subcellular poly(A)⁺ RNA. Poly(A)⁺ RNA was isolated from whole cells or from subcellular fractions and analyzed by RNA transfer blotting and hybridization with whole genomic probe as described in the text. (A) Analysis of whole cell and cytoplasmic poly(A)⁺ RNA. Track 1, 10 μ g of whole cell poly(A)⁺ RNA; track 2, 10 μ g of cytoplasmic poly(A)⁺ RNA. (B) Analysis of whole cell, polysomal, and postpolysomal poly(A)⁺ RNA. A cytoplasmic extract was prepared from 10⁸ VX-7 cells and fractionated into polysomes and a postpolysomal supernatant as described in the text. RNA was extracted from the polysomes directly and from the postpolysomal supernatant after the addition of polysomes prepared from 10⁸ HeLa cells. Poly(A)⁺ RNA was isolated from the subcellular fractions, and 50 μ g was obtained from both fractions. Poly(A)⁺ RNA was also isolated from whole-cell RNA extracts. Poly(A)⁺ RNA isolation and analysis was as described for (A). Track 1, 5 μ g of whole-cell poly(A)⁺ RNA; track 2, 10 μ g of polysomal poly(A)⁺ RNA; track 3, 20 μ g of postpolysomal poly(A)⁺ RNA.

Despite the degradation problem, the cell fractionation experiments permit us to conclude that the major transcripts are transported into the cytoplasm and that at least one should be translated.

The low abundance of viral transcripts in Shope virus-induced domestic rabbit tumors makes mapping of the RNA very difficult. This is illustrated by the fact that viral transcripts could not be detected reproducibly in RNA transfer blot analyses of unfractionated cellular RNA (20 μ g of RNA per track), even with high-specific-activity probes (1.0×10^9 cpm/ μ g DNA), and by the fact that the amount of poly(A)⁺ RNA (10 and 20 μ g) analyzed in Fig. 1 is about 1,000 times more than was needed in comparable blots to detect immunoglobulin light-chain mRNA in myeloma cell poly(A)⁺ RNA (kindly provided by E. Choi). Since 10 μ g of poly(A)⁺ RNA represents the material ob-

tained from 1 g of tumor, extensive analyses are not possible with the RNA isolated from average 5- to 10-g tumors. Therefore, mapping studies were performed with the VX-7 cell line.

The mapping of viral RNA in VX-7 cells was performed with poly(A)⁺ RNA isolated from whole cells. In these experiments, two sets of subgenomic probes were hybridized to RNA transfer blots. The first set represented the four Shope virus-specific fragments generated by *Bgl*II-*Eco*RI double digestion of Shope-pBR322 recombinant plasmid (17). The second set of probes were Shope DNA fragments obtained by *Msp*I digestion of the recombinant DNA or by redigestion with *Msp*I of *Bgl*II-*Eco*RI fragments. The map of the *Bgl*II and *Msp*I sites is shown in Fig. 3 (top). Subgenomic fragments separated by agarose gel electrophoresis and recovered by electroelution were labeled by nick translation (11). The probes were tested for purity by hy-

FIG. 3. Mapping of Shope virus-specific transcripts by hybridization of RNA transfer (Northern) blots with subgenomic probes. The top of the figure shows a map of *Eco*RI, *Sal*I, *Bgl*II, and *Msp*I sites in cloned Washington B strain Shope (rabbit) papilloma virus DNA (see text). (A) Hybridization of subgenomic probes to DNA transfer (Southern) blots of restriction enzyme-digested Shope-virus DNA. In the group on the left, fragments generated by *Bgl*II-*Eco*RI double digestion were separated on 1% agarose gels, transferred to cellulose nitrate filters (14), and hybridized (16) with various probes; track W contained whole Shope DNA probe and tracks IIIa, II, I, and IIIb represent hybridizations with the four subgenomic probes generated by *Bgl*II-*Eco*RI double digestion. In the group on the right, fragments generated by *Msp*I digestion were separated on 1.5% agarose gels, and blots were prepared and hybridized with various probes; track W contained whole Shope DNA probe and tracks 5, 8, 6, 4, and 1 represent hybridizations with subgenomic probes generated by *Msp*I digestion. The sizes of the molecular-weight markers indicated from top to bottom are 4.2, 2.2, 1.8, 1.35, 1.08, 0.87, 0.60, 0.28, and 0.23 kb. (The markers on the left correspond to the left half of the panel, tracks W to IIIb; those on the right correspond to the right half of the panel, tracks W to 1.) (B) Hybridization of subgenomic probes to RNA transfer (Northern) blots of total cellular VX-7 RNA. The RNA transfer blots were prepared and hybridized to the subgenomic probes characterized in (A). The molecular-weight markers indicate the positions of 18S and 28S rRNA. The map position of the transcripts is indicated below the restriction map of the Shope DNA (top). The arabic figures refer to restriction fragments generated by *Msp*I digestion. The dots indicate that the exact beginning and end of the transcripts is not known. The size of a 1-kb marker is indicated.

bridization to Southern blots of *Bgl*III-*Eco*RI- or *Msp*I-digested Shope DNA (Fig. 3B). Three of the *Bgl*III-*Eco*RI fragments hybridized to both transcripts, and fragment II did not hybridize to either of the transcripts (Fig. 3B). The genomic location of the transcripts was narrowed further by hybridizations with *Msp*I fragments 1, 4, 5, 6, and 8. Of these fragments, 4 and 6 hybridized, and 1, 5, and 8 did not (Fig. 3B). Taken together with the positive hybridization observed with the *Bgl*III-*Eco*RI fragments IIIa and IIIb, these results suggest that the transcripts within these two fragments originate predominantly from *Msp*I fragments 7a and 7b and possibly from fragment 10. These experiments also suggest that the major transcripts are colinear since, within experimental error, 1.9 kb (the sum of *Msp*I fragments 4, 6, 7a, and 7b) is the size needed to encode the larger transcript. We cannot, however, exclude the possibility that the two transcripts are of opposite polarity, unless it is assumed that the maximal value of 11% observed in single-phase liquid hybridization reflects complete hybridization of probes to both transcripts. The mapping experiments also indicate that both transcripts must contain at least one splice because in some experiments (data not shown) the larger minor transcripts (but never the major transcripts) were detectable in hybridizations with *Msp*I fragment 1. Thus, this splice must eliminate an intron which spans *Msp*I fragment 1.

DISCUSSION

The results reported here show that non-virus-producing benign and malignant tumors induced by the Shope virus contain major transcripts of 1.3 and 2.0 kb, which are present at low abundance. In one analysis (Fig. 1, track 5), there is some indication that the 2.0-kb band is resolved into two bands. In general, however, the major bands could not be further resolved when analyzed on 1.1 or 1.5% agarose gels, and we estimated that if two RNA species of similar intensity were present their size could differ by not more than 100 base pairs in the 1.3-kb band and by not more than about 150 base pairs in the 2.0-kb band. In the benign tumor, the larger transcript appears to be more common, whereas the reverse situation is found in the malignant tumor induced by the virus directly, in the transplantable carcinoma (data not shown), and in the VX-7 cell line. Cell fractionation of the VX-7 cells indicates that both RNA species are transported to the cytoplasm, and at least one becomes polysome associated. Furthermore, minor, larger transcripts of 3.5 to 4.8 kb that were detectable in some analyses of total cellular RNA (Fig. 3B, track IIIa) were never seen in the cytoplasmic fraction. In contrast, poly A⁺

RNA, although not resolved into specific bands, contained a considerable fraction of virus-specific RNA larger than 2.0 kb (data not shown). Finally, mapping studies of the viral transcripts in the VX-7 cells indicated that both transcripts are spliced and colinear.

The presence of the same-sized transcripts in virus-induced benign and malignant tumors, as well as their continued presence in a cell line derived from the VX-7 carcinoma (which was established over 30 years ago), strongly suggest that the transcripts, and most likely their translation products, are essential for the maintenance of the tumors. Furthermore, it is interesting to note that the state of the DNA (all integrated in the cell line and predominantly extrachromosomal in carcinomas induced by the virus directly) seems not to affect the nature of the transcripts.

As indicated, no qualitative difference was found between transcripts of benign and malignant tumors. However, some evidence for a quantitative difference was obtained, suggesting some change in transcription during tumor progression. Surprisingly, in bovine papilloma virus type 1-induced malignant hamster tumors, only 1.3-kb transcripts were found (5), and this could indicate that a predominance of 1.3-kb transcripts is characteristic of the malignant state of papilloma virus-induced tumors. Furthermore, the suggestion that tumor progression is paralleled by some changes in viral DNA transcription is supported by our finding that viral DNA methylation in carcinomas is different from that in papillomas (F. O. Wettstein and J. G. Stevens, manuscript in preparation).

Our analysis of subcellular fractions revealed that some virus-specific RNA is polysome associated, and this suggests that some viral transcripts are translated. A degradation problem encountered during polysome preparation did not permit us to determine whether one or both of the cytoplasmic transcripts are polysome associated. Both transcripts would, however, be adequate in size to code for a 29-kilodalton protein immunoprecipitated from labeled VX-7 cell extracts (Stevens and Wettstein, unpublished data). The analysis of viral RNA separated into a nuclear and a cytoplasmic fraction indicated that the minor 3.5- to 4.8-kb transcripts observed in some analyses of total cellular RNA may be of nuclear origin. This is suggested because virus-specific sequences larger than 2.0 kb represent a considerable fraction of the virus-specific nuclear RNA, whereas no such sequences were detected in the cytoplasmic fraction. Of course, our data do not indicate if a precursor-product relationship exists between the larger, minor transcripts and the smaller, major transcripts.

The mapping studies indicated that both major transcripts are spliced and are most likely colinear. Our data do not prove directly that both viral RNAs are transcribed from the same DNA strand and are thus colinear. This conclusion is based on the assumption that the maximal level of hybridization (11%) observed in single-phase liquid hybridization of RNA isolated from the transplantable tumor as well as from the tumor-derived cell line (7a) represented complete hybridization of both major transcripts.

The ongoing investigations of the state of the viral genome (19) and its expression in benign and malignant tumors induced by the Shope virus continue to show quantitative rather than qualitative differences between the two types of tumors. Clearly, it cannot be stated yet whether these differences are causally related to tumor progression.

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