MOLECULAR AND CELLULAR BIOLOGY, Mar. 1989, p. 925–934
0270-7306/89/030925-10$02.00/0

Vol. 9, No. 3

Cell-Heritable Stages of Tumor Progression in Transgenic Mice Harboring the Bovine Papillomavirus Type 1 Genome

MARIA SIPPOLA-THIELE,[1]† DOUGLAS HANAHAN,[2]‡ AND PETER M. HOWLEY[1]*

Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda, Maryland 20892,[1] and Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724[2]

Received 9 September 1988/Accepted 28 November 1988

Tumorigenesis of dermal fibroblasts in a line of transgenic mice carrying the BPV-1 genome was found to involve distinct proliferative stages. Cell cultures derived from normal skin, from benign proliferative fibromatoses, and from malignant fibrosarcomas each evidenced distinguishable, cell-heritable characteristics. The latent viral genome was transcriptionally inactive in normal-appearing skin and was activated in the dermal fibromatoses. Fibrosarcoma cells grew continuously in culture, formed domelike foci, and had a more rounded, anaplastic appearance. Independent cultures derived from the fibromatoses varied in their proliferative characteristics, which correlated well with the levels of viral gene expression. In contrast, progression to malignancy was not accompanied by a further increase in transgene activity, which strongly implicated cellular genetic changes in the later stages of tumorigenesis.

The epidemiology of cancer indicates that it involves multiple steps and that the conversion of a normal cell to a neoplastic one is likely to be a consequence of a series of separable events (7, 13, 14). Over the past decade, a number of cellular and viral genes have been identified which, by themselves or in various combinations, are able to transform established cell lines or primary cells to a malignant phenotype in tissue culture. Studies have indicated that mutations which either directly affect the coding sequences of some cellular oncogenes or alter their regulated expression may be involved in specific steps of cancer progression in humans and in experimental animals (3). Additional genetic loci can suppress the cancerous phenotype of a cell, and the loss of these loci appears to play an important and possibly necessary role in some types of neoplasia. It is implicit that these losses involve the elimination of a gene (or genes) whose products function to counteract some aspect of cell proliferation, tumor progression, or both. It has not yet been possible to separate the individual stages involved in tumor progression, and it has been difficult to identify at which stages individual viral and cellular oncogenes may play a role.

The papillomaviruses are a group of DNA viruses associated with benign epithelial tumors in a variety of higher vertebrates. There is a subgroup of the papillomaviruses, of which bovine papillomavirus type 1 (BPV-1) is the prototype, which induce fibropapillomas characterized by squamous epithelial as well as proliferative dermal fibroblastic components. Some of the papillomaviruses have been associated with naturally occurring carcinomas in their normal hosts (reviewed in reference 10). In the cancers associated with these papillomaviruses, progression to malignancy occurs from the benign papillomas after a latent period of variable length, and this malignant progression is usually associated with external cofactors.

Transgenic mice are providing a new approach with which to address the molecular genetics of the multiple stages in cancer progression (9). A number of oncogenes have been established in transgenic mice. Many of these transgenic lineages heritably develop specific types of tumors which are characterized both by the nature of the oncoproteins that elicit them as well as by the regulatory elements that control them (19). Among these is a line of transgenic mice which carry the BPV-1 genome at a single random chromosomal location. Previously, it was shown that transgenic mice in this lineage develop two characteristic pathologies: abnormal skin, which is characterized by a hyperplasia of dermal fibroblasts; and protuberant tumors, which have a primary component of dermal fibroblastic cells (15). In this previous study, it was shown that the BPV-1 genomes are stably integrated in the normal tissues of the mice but are present as extrachromosomal plasmids in the abnormal skin tissues and in the dermal fibroblastic tumors. There is an unusually long latency associated with the development of abnormal pathologies, each first appearing at about 8 to 9 months of age.

A detailed analysis of the cellular and molecular biology of the pathologic conditions associated with BPV 1.69 transgenic mice is presented here. Cultures were derived from normal skin, from areas of abnormal skin, and from protuberant tumors, and analyses of these cultures revealed that the characteristics of these lesions are cell heritable. The data indicate that expression of the BPV-1 genome occurs only in the proliferative lesions and that viral gene expression alone is not sufficient for progression to malignancy. These observations suggest that additional cellular events are required for papillomavirus-associated neoplastic progression in transgenic mice.

MATERIALS AND METHODS

Cell cultures. Cells were cultured and maintained in Dulbecco modified Eagle medium complete with penicillin (100 U/ml), streptomycin (100 μg/ml), and heat-inactivated fetal calf serum (10%). The cell cultures were established from skin and tumor tissues of several independent BPV 1.69 transgenic mice. Tissues were minced, placed in a small amount of Dulbecco modified Eagle medium containing 10% fetal calf serum into culture dishes for attachment, and subsequently expanded. The nomenclature for the cell cul-

* Corresponding author.
† Present address: Department of Pediatrics, University of Michigan, Ann Arbor, MI 48109.
‡ Present address: Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, CA 94143.

tures and tissues includes either the number of the transgenic mouse or the designation 2d for 2-day-old, 14w for 14-week-old, or 16w for 16-week-old. The abbreviations used are T (tumor), F (abnormal skin with fibromatosis), AF (abnormal skin with aggressive fibromatosis), NS (normal skin), and IO (internal organs). Cell cultures are designated ^{CC}, and established cell lines are designated ^{CL}.

Growth curve. Cells were seeded on 60-mm plates at a density of 10^4 per plate and grown in Dulbecco modified Eagle medium. Duplicate plates were counted for each time point.

Growth in low serum concentrations. Cells (5×10^4) were seeded on 60-mm plates and grown in 0.5, 1, 5, or 10% serum concentrations. Medium was changed every 72 h, and growth was determined over a 1-month period.

Tumorigenicity studies. Tumorigenicity studies were carried out in two strains of athymic nude mice (NIH BALB/c and Swiss HO) and in syngeneic mice (B6D2F1/J, a cross of C57BL/6J and DBA/2J). Cells (10^6 or 10^7) were injected subcutaneously, and the time of appearance of tumor formation was noted. The cells were from between passages 4 and 10, as specified.

Extraction and analysis of cellular DNA and RNA. DNA and RNA from frozen tissues were extracted after being pulverized in a Mikro-Dismembranator II (B. Braun, Melsungen, Federal Republic of Germany). Genomic DNA was isolated from pulverized tissue or from lysed cultured cells by a method described previously (8). Restriction endonuclease digestion, gel electrophoresis, and filter hybridization analysis of DNAs were performed by standard methods (17). Total-cell RNA was extracted from pulverized tissue or from cultured cells by using the guanidinium isothiocyanate method described by Chirgwin et al. (4).

Total-cell RNAs were electrophoresed through 1.2 to 1.4% agarose gels in the presence of 2.2 M formamide (16). The RNA was transferred to GeneScreen by capillary blotting in 25 mM sodium phosphate (pH 6.5) for 12 h. RNA was bound to the nylon membrane by UV irradiation (5). Hybridization buffers and washing solutions were prepared according to a method of Church and Gilbert (5). The hybridization probes for Northern (RNA) blot analyses were generated by using universal primer and primer extension labeling as described by Feinberg and Vogelstein (6).

Immunoprecipitation of E5 protein. Subconfluent cells in 10-cm^2 tissue culture plates were labeled with 0.5 mCi of [^{35}S]cysteine and 0.5 mCi of [^{35}S]methionine in Dulbecco modified Eagle medium depleted of cysteine and methionine. The cells were washed with phosphate-buffered saline and scraped into RIPA buffer (20 mM MOPS [morpholinepropanesulfonic acid] buffer, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 1% sodium dodecyl sulfate [pH 7.0]), and immunoprecipitations were carried out with antibodies to the E5 protein as described by Schlegel et al. (22) except that the immunoprecipitations were repeated twice to reduce background.

RESULTS

Dermal proliferative abnormalities in BPV 1.69 mice. The histopathology of approximately 20 BPV 1.69 transgenic mice was examined to further characterize the stages involved in tumor progression in these mice. No histologic abnormalities were noted in mice less than 5 months of age in which there were no gross skin lesions. In areas previously described as abnormal skin but without protuberant tumors, the pathology was primarily associated with dermal fibroblasts. There was, however, a spectrum of pathology depending on the extent of dermal fibroblastic proliferation, with some lesions demonstrating only a sparse but diffuse proliferation of cells and others demonstrating a dense dermal proliferation which extended into the subcutaneous tissues. It is appropriate to use the term fibromatosis to describe this pathology, since the proliferative cell is the dermal fibroblast. Similarly, aggressive fibromatosis describes the densely proliferative lesions. There was atrophy of the overlying epidermis, with loss of dermal appendages such as sweat glands and hair follicles in the sections of the fibromatoses, which accounted for the gross abnormal appearance of the skin.

The protuberant tumors varied in appearance but were basically fibroblastic in nature and most likely derived from the dermal fibroblast. Some of the tumors were composed of interlacing bundles of spindle-shaped fibroblasts, characteristic of a low-grade fibrosarcoma. Others were still fibroblastic in nature but had a more anaplastic appearance, often with bizarre nuclei.

Cell cultures derived from skin tissues. To further characterize the stages of cellular proliferation and tumor progression noted in the BPV 1.69 transgenic mice, cells were cultured from the dermal fibroblasts of young transgenic mice with apparently normal skin, from the dermal fibroblasts of the abnormal skin of affected mice, and from the dermal fibroblastic tumors. The initial growth characteristics of the cells were quite interesting. Cell cultures established from the dermal fibroblastic tumors of several mice (007, 855, 851, 3195, and 3142), exhibited a phenotype characteristic of transformed cells. The cells proliferated rapidly and formed dome-shaped foci of non-contact-inhibited cells (Fig. 1D).

In contrast, cultures established from areas of abnormal skin, which maintained the topology of the skin, did not show this focus-forming phenotype. A representative of one class of cultures, which was established from an area of aggressive fibromatosis from mouse 007 (007-AF^{CL}), is shown in Fig. 1C. These cells were not contact inhibited and grew to high density but did not form foci. Two other cell lines, each derived from an area of fibromatosis in a separate BPV 1.69 mouse, had a similar phenotype. The histologic appearance of the lesions from which these cultures were derived was that of a dense dermal fibroblastic proliferation characteristic of aggressive fibromatosis. A second class of cell lines established from the dermal fibroblasts in areas of fibromatosis grew poorly in culture and were contact inhibited (1394-F^{CC}, 3147-F^{CC}, and 2250-F^{CC}) (Fig. 1B). Cultures of dermal fibroblasts from the normal skin of three independent transgenic mice of ages 2 days, 14 weeks, and 16 weeks had characteristics of primary cultures (Fig. 1A). These cells were contact inhibited, grew poorly, and could not be continually passaged.

To more accurately assess the growth characteristics of these cultures, cells were seeded at a density of 10^4 per plate and counted at 24-h intervals. The growth curves (Fig. 2) show the rapid rate of proliferation of the tumor line 007-TU^{CL}, which was characteristic of each of the tumors analyzed. The doubling time for this culture was 39 h. The 007-AF^{CL} cell line grew rapidly and was indistinguishable in its growth characteristics from the tumor cell lines (see below). Most of the dermal fibroblastic cultures derived from areas of fibromatosis had growth characteristics similar to those of 1394-F^{CC}, which had a doubling time of 69 h. Normal skin fibroblasts cultured from 2-day-old BPV 1.69 mice, 2d-NS^{CC}, did not grow well when plated at a density of

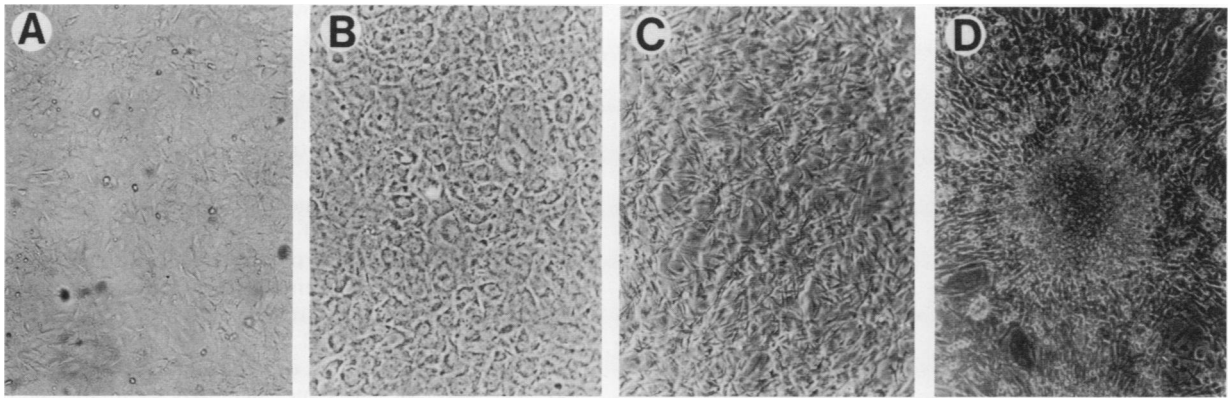

FIG. 1. Morphology of cultured cells derived from dermal fibroblasts (p4) from a 2-day-old BPV 1.69 mouse (A), an area of fibromatosis from mouse 3147 (p5) (B), an area of aggressive fibromatosis from mouse 007 (p4) (C), and a fibrosarcoma from mouse 3137 (D). Magnification: A and B, ×250; C and D, ×100.

10^4 cells per 60-mm plate. A similar growth pattern was noted for primary skin fibroblasts cultured from the normal-appearing skin of 14-week-old and 16-week-old transgenic mice.

Representative cell lines were also examined for ability to grow under low-serum conditions. At serum concentrations of 0.5 and 1%, only 007-AF^{CL} and the tumor cell lines 007-TU^{CL} and 851-TU^{CL} were able to grow. The slowly proliferating fibromatosis cell cultures (1394-F^{CC}) and cultured cells derived from normal skin (2d-NS^{CC}) grew very poorly even in 5% serum.

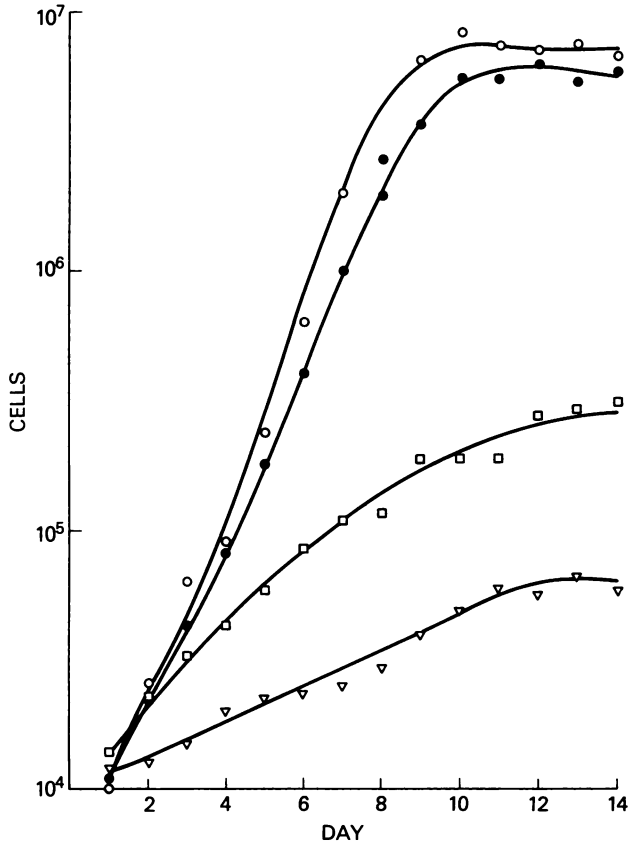

FIG. 2. Growth curve of BPV 1.69 transgenic mice cell cultures. Shown are the tumor cell line 007-TU^{CL} at passage 5 (○), a cell line (007-AF^{CL}) derived from an area of aggressive fibromatosis at passage 5 (●), a cell culture derived from an area of fibromatosis (1394-F^{CC}) at passage 4 (□), and a dermal fibroblast culture derived from the normal skin of a 2-day-old mouse (2d-NS^{CC}) at passage 3 (▽). Cell numbers were determined in duplicate 60-mm plates for each cell line.

Tumorigenicity was tested by injecting 10^6 or 10^7 cells subcutaneously into athymic nude mice and into 4-week-old syngeneic B6D2F1/J mice (Table 1). Neither the 1394-F^{CC} nor the 2d-NS^{CC} cell culture was tumorigenic, even after 16 weeks of observation. At 3 weeks, both the aggressive fibromatosis cell line 007-AF^{CL} and the fibrosarcoma cell line 007-TU^{CL} produced large tumors in either immunosuppressed or immunocompetent hosts at either cell concentration. Despite the clear in vivo differences between aggressive fibromatoses and the protuberant fibrosarcomas, it is remarkable that there were few distinguishing characteristics noted during in vitro culture. Indeed, this finding points out one of the limitations of cell culture in assessing the phenotype of malignant cells and indicates an additional positive aspect of using transgenic mice.

RNA analysis. The transcriptional characteristics of the BPV-1 genome when maintained in the mouse germ line have not been previously assessed. Among the possibilities are tissue-specific expression in skin tissue, tissue-specific activation in skin lesions, and general expression in many tissues. To address these various possibilities, RNA from skin lesions and from the cell cultures described above were analyzed for BPV-1 transcription. RNA was extracted directly from the tissues of several independent mice. No BPV-1-specific transcription was detected in the skin tissues of 16-week-, 14-week-, and 2-day-old transgenic mice (Fig.

TABLE 1. Characteristics of skin cell cultures derived from BPV 1.69 mice

Cells	Pathology	Tumorigenicity[a]		Growth in low serum concn
		Nude	Syngeneic	
007-TU^{CL}	Fibrosarcoma	15/15	2/5	+
007-AF^{CL}	Aggressive fibromatosis	15/15	5/5	+
1394-F^{CC}	Fibromatosis	0/5	0/5	−
2d-NS^{CC}	Normal	0/5	0/5	−

[a] Determined at 6 weeks after subcutaneous injection of cells. The 1394-F^{CC} and 2d-NS^{CC} cells remained nontumorigenic even after 16 weeks of observation.

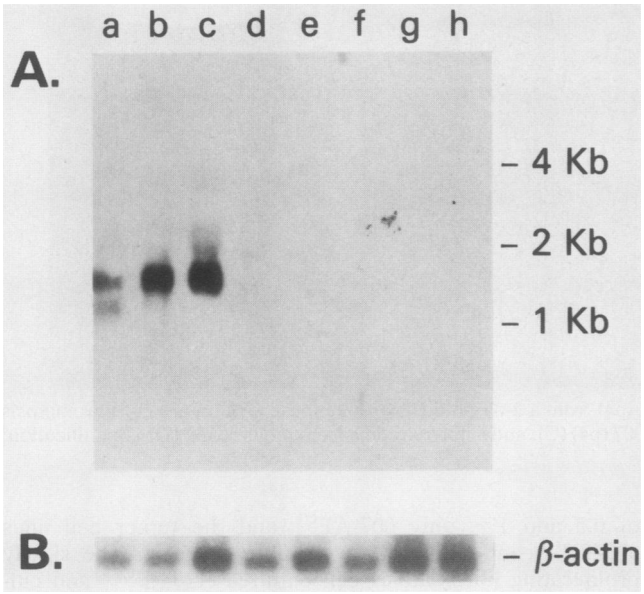

FIG. 3. RNA analysis of total cellular RNA from transgenic mouse tissues. (A), Total-cell RNAs analyzed by Northern blot hybridization, using a BPV-1 DNA probe. RNAs were isolated from two independent mouse tumors, 855-TU (lane a) and 007-TU (lane b); an aggressive fibromatosis (mouse 007) (lane c); a fibromatosis (mouse 3147) (lane d); normal skin from a 16-week-old mouse (lane e), a 14-week-old mouse (lane f), and a newborn mouse (lane g); and liver involved with lymphoma (mouse 860) (lane h). As a quantitation control for RNA, the blot was stripped and rehybridized to a beta-actin probe (B).

3, lanes e through g). In addition, the internal organs, including brain, liver, gastrointestinal tract, stomach, lung, pancreas, heart, and kidneys (analyzed from mice 007, 855, and 851), did not have detectable levels of BPV-1-specific transcripts (data not shown). Finally, analysis of a lymphoma (860-Ly) detected in one transgenic mouse in the colony revealed no viral transcripts (Fig. 3, lane h). The BPV-1-specific transcripts were detected only in the skin tumors and in areas of dermal fibromatosis (Fig. 3). All tumors expressed BPV-1 transcripts at levels similar to that seen in the cell line ID13, which is a C127 cell line transformed by BPV-1 (data not shown). Viral transcription is complex in BPV-1-transformed cells in that a series of mRNAs varying in size from about 1.1 kilobases (kb) up to 4.0 kb are produced from the transforming region of the genome. This complexity results from the use of at least five promoters in transformed rodent cells as well as complex patterns of RNA splicing of the primary transcripts (2, 24, 25). The level of virus-specific mRNAs in the aggressive fibromatosis tissue (007-AF) was equivalent to those of the tumors, and the patterns of bands visible by Northern analysis appeared similar as well. The tissues of abnormal skin with nonaggressive fibromatosis contained levels of BPV-1-specific RNA about 5- to 10-fold lower than those observed in the tumors and aggressive fibromatosis. This finding was quantitated by densitometric scanning of different exposures of autoradiograms that had been normalized to the amounts of beta-actin expression in the tissues (Fig. 3B).

As part of an evaluation of the relevance of cultured cells to the pathologic tissues from which they were derived, BPV-1-specific transcripts in tissues and in the cultured cells derived from those tissues were compared. The patterns and levels of BPV-1-specific transcripts in the cells in culture mirrored the patterns and levels seen in tissue biopsies. Comparison of the viral transcripts from tumor tissue (007-TU) and the cell line derived from that tissue (007-TU^{CL}) is shown in Fig. 4. Cultures from the skin of unaffected mice revealed no evidence of BPV-1 expression (Fig. 4B, lanes a and b). Viral transcription was easily detected in cultures of the fibroblastic tumors (lanes c through e) and in cultures derived from the abnormal skin tissues with fibromatosis

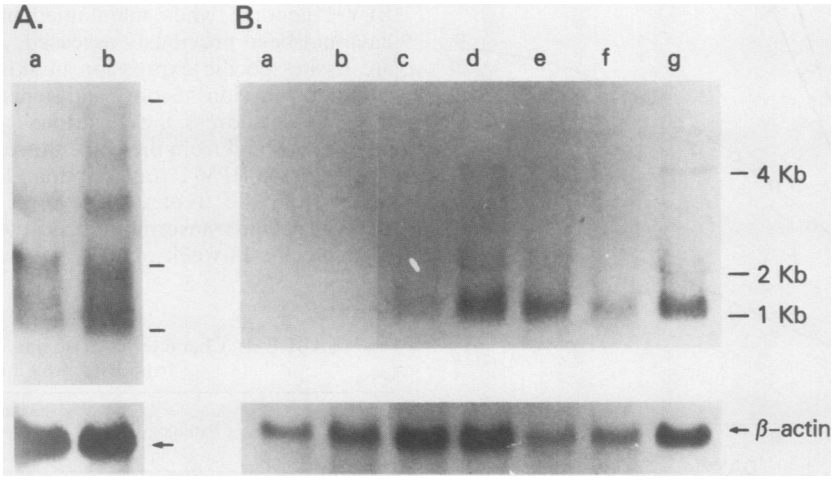

FIG. 4. BPV-1-specific RNA in cell cultures derived from BPV 1.69 mice. (A) Northern analysis comparing RNA extracted directly from tumor 007 (lane a) and cultured cells derived from that tumor (p4) (lane b). Hybridization was performed with a ^{32}P-labeled BPV-1 DNA probe. Subsequently, the RNA was rehybridized with a beta-actin probe as a quantitation control (arrow). (B) Northern analysis of RNAs extracted from cell cultures established from transgenic mouse tissues. Cell cultures were derived from a 2-day-old mouse, 2d-NS^{CC} (passage 5) (lane a); a 14-week-old mouse, 14w-NS^{CC} (passage 5) (lane b); fibrosarcoma from mouse 851 (passage 5) (lane c), mouse 855 (passage 7) (lane d), and mouse 007 (passage 8) (lane e); an area of fibromatosis from mouse 1394, 1394-F^{CC} (passage 5) (lane f); and an area of aggressive fibromatosis from mouse 007, 007-AF^{CC} (passage 7) (lane g). Hybridization was carried out with a ^{32}P-labeled BPV-1 DNA probe, and the blot was rehybridized with a beta-actin probe as a quantitation control.

TABLE 2. Analysis of BPV-1 DNA and RNA in BPV 1.69 mice

Tissue	Cell culture[a]	DNA[b]	RNA[c]
Tumor			
007-TU	007-TU^{CL}	e	+
855-TU	855-TU^{CL}	e	+
851-TU	851-TU^{CL}	e	+
1395-TU	1395-TU^{CL}	e	+
3142-TU	3142-TU^{CL}	e	+
860-TU	NE[d]	e	+
860-Ly	NE	i	−
Aggressive fibromatosis			
007-AF	007-AF^{CL}	e	+
3142-AF	3142-AF^{CL}	e	+
61U1-AF	61U1-AF^{CL}	e	+
Fibromatosis			
1394-F	1394-F^{CC}	e	+
3147-F	3147-F^{CC}	e	+
2250-F	2250-F^{CC}	e	+
XX15-F	XX15-F^{CC}	e	+
4216-F	4216-F^{CC}	e	+
Normal skin			
2d-NS	2d-NS^{CC}	i	−
14w-NS	14w-NS^{CC}	i	−
16w-NS	16w-NS^{CC}	i	−
Internal organs			
007	NE	i	−
855	NE	i	−
851	NE	i	−
1394	NE	i	−

[a] Cultured cells were analyzed between passages four and ten.
[b] DNA was examined by Southern blot analysis, using enzymes that do not cleave within the transgene. e, Extrachromosomal plasmid viral DNA detected; i, only the integrated transgene detected.
[c] BPV-1 transcription was assessed by Northern blot analysis. Positive and negative hybridization are indicated by + and −, respectively.
[d] NE, Not examined.

(Fig. 4B, lanes f and g). The levels of transcription correlated with the amount of extrachromosomal DNA in the fibromatosis tissues (see below).

A survey of the RNA analyses carried out on several mice is shown in Table 2. There was a complete correlation between the presence of extrachromosomal BPV-1 DNA and the expression of the viral RNA. We also investigated the possibility that the late genes were being expressed. Four samples, 007-TU, 007-AF, 1394-F, and 14w-NS, were analyzed by using a late open reading frame probe, with bovine wart RNA as a positive control (2). In this analysis, late gene transcription was not detected in any of the samples derived from these transgenic mice (data not shown).

In summary, the BPV-1 genome was transcriptionally inactive in all normal tissues, including skin. It therefore appears that there is a transcriptional activation of the latent viral genome, which strictly correlates with the development of dermal fibroblastic proliferation and eventually of skin tumors. The cell lines derived from different skin lesions showed transcriptional levels similar to those of the primary tissue samples from which they were derived, which supports their identification and use as representative in vitro models of the distinct tissue pathologies.

Presence of the BPV-1 oncoproteins E5 and E6. There are two oncoproteins encoded by the BPV-1 genome, as identified by assays which involve morphological transformation of cultured cells (reviewed in reference 11). Given that the BPV-1 genome is acting like a tissue-specific oncogene in transgenic mice, the possible involvement of the BPV-1 oncoproteins in the transformation process was investigated. The two oncogenes encoded by BPV-1 are called E5 and E6 (21, 25), and antibodies to each of these proteins have recently been described (1, 22). Cultured cells representing each of the pathologies described and cells from normal skin were each metabolically labeled with ^{35}S and assayed by immunoprecipitation for the presence of E5. The E5 protein was detected in each of the three independent tumor cell lines examined and in two cell lines from aggressive fibromatosis (Fig. 5). The levels of E5 protein were very low and were essentially undetectable in cultured cells from areas of nonaggressive fibromatosis (Fig. 5) as well as in normal skin fibroblasts.

By using a similar immunoprecipitation assay, small amounts of the E6 protein were also detected in cultured

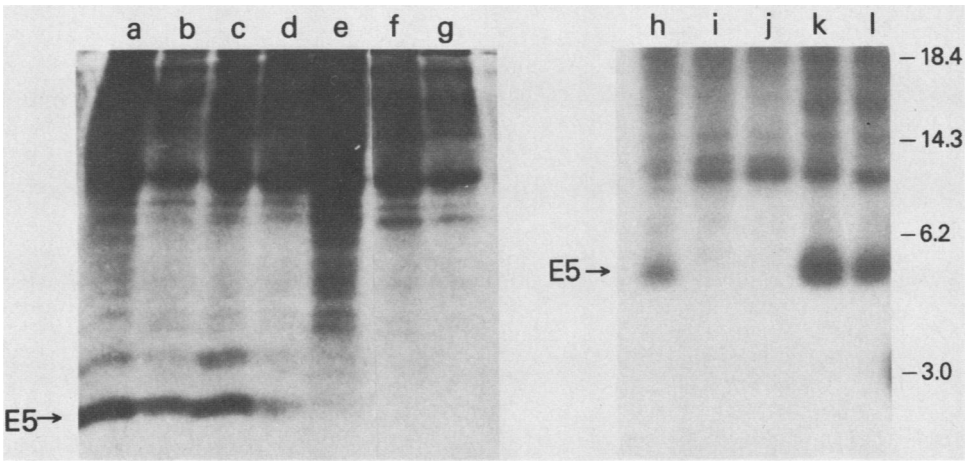

FIG. 5. Analysis of E5 oncoprotein levels. Cultured cells from transgenic mice tissue were metabolically labeled with [^{35}S]methionine and assayed by immunoprecipitation for E5. The immunoprecipitates were analyzed on 15% sodium dodecyl sulfate-polyacrylamide gels, followed by autoradiography. Lanes: a and h, positive control of C127 cells transformed by a plasmid in which the E5 oncoprotein is expressed from the murine sarcoma virus long terminal repeat; g, negative control of dermal fibroblasts from non-BPV transgenic mice; i, dermal fibroblasts from the normal-appearing skin of a 16-week-old BPV 1.69 mouse; b, k, and l, from tumor cell lines from mice 3142, 851, and 007, respectively; c and d, from the aggressive fibromatosis cell lines from mice 3142 and 61U1, respectively; e, f, and j, from nonaggressive fibromatoses from mice XX15, 4216, and 1394, respectively.

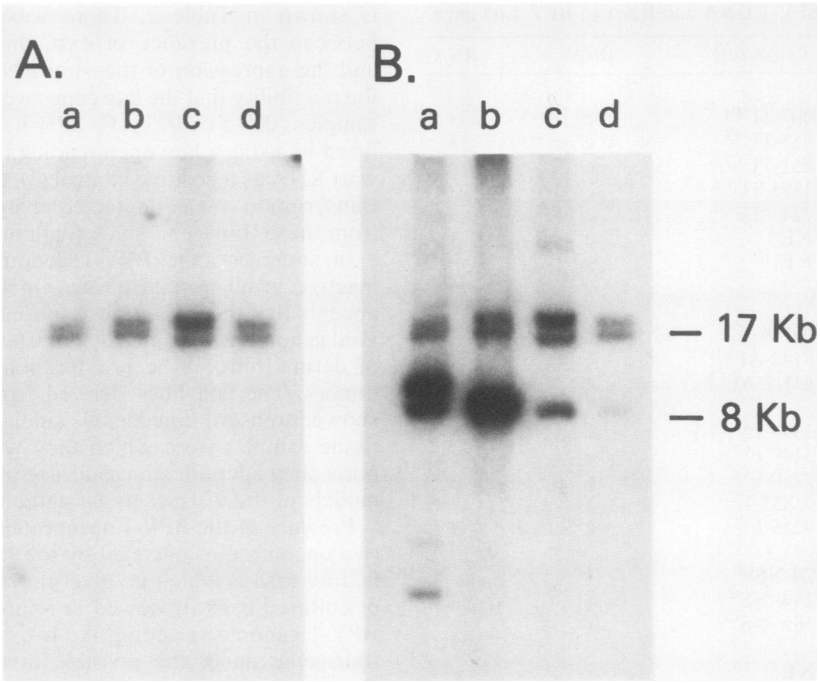

FIG. 6. Analysis of the BPV transgenome in BPV 1.69 mouse tissues. Genomic DNA was isolated from a tumor of mouse 007 (lane a), a lesion with aggressive fibromatosis from mouse 007 (lane b), a fibromatosis from mouse 3147 (lane c), and the kidney of mouse 2250 (lane d). Tissue DNAs were digested with *Xba*I to linearize the transgenome, and the samples were electrophoresed on a 0.7% agarose gel and transferred to nitrocellulose. (A) Hybridization with pBR322; (B) hybridization with BPV-1 DNA. Extrachromosomal BPV-1 DNA could easily be detected in the abnormal skin and tumor samples (15). The faint 8-kb band noted in lane d of panel B was not due to cleavage of an extrachromosomal 8-kb circular molecule but rather to a germ line rearrangement of an integrated concatamerized plasmid as previously described (15).

tumor cells and in cultured cells derived from an aggressive fibromatosis (data not shown). As with E5, the E6 protein was undetectable in fibromatosis cell lines that grew poorly in culture. Since E6 is normally very difficult to detect in most BPV-1-transformed cell lines, this result most likely reflects a failure to detect rather than an absence of the viral oncoprotein. In sum, the E5 and E6 oncoproteins were detectable in cells derived from aggressive fibromatoses and from the tumors, which indicated a correlation between a high proliferative rate of the cells and the levels of the viral oncoproteins. Although the immunological reagents currently available are not sensitive enough to detect the presence of the proteins in nonaggressive fibromatoses, the fact that there was discernible viral transcription in the lesions suggests that low levels of E5 and E6 were also likely to be present. It is notable that no difference in E5 and E6 levels could be seen in comparisons of the aggressive fibromatoses and the tumors, which suggests that increased levels of the oncoproteins are not completely responsible for this step in progression.

State of viral DNA in BPV 1.69 transgenic mouse tissues and cell cultures. There is a complete correspondence between the development of skin lesions and the appearance of extrachromosomal copies of the BPV-1 genome. However, it is not clear whether the generation of extrachromosomal BPV-1 DNA is causal to the tumorigenesis process, nor is it clear as to the mechanism by which these plasmids are produced. Since the excision and gene amplification of BPV-1 DNA could be relevant to the latency and activation, as well as to the progression, of the transformed state, the analysis of BPV-1 DNA in normal and abnormal tissues was extended. Furthermore, it was important to establish the relationship of the BPV-1 DNA in the pathologic tissues and in the cell lines derived from the tissues.

DNAs were isolated directly from a variety of tissues from the transgenic mice and from cell cultures established from these tissues. The tissues studied were from the tumors, from the abnormal skin areas of fibromatosis, and from the skin of mice with no abnormalities. Total genomic DNA was digested with the restriction enzyme *Xba*I, which cuts once within the transgenome and consists of a recombinant plasmid containing a partial tandem duplication of BPV-1 DNA in pBR322 (15). By using a pBR322 DNA probe (Fig. 6A), the procaryotic sequences could be detected only associated with the integrated copies of the transgene in the mouse genomic DNA. The integrated sequences were detected as a pair of bands approximately 17 kb in size, as originally shown by Lacey et al. (15). The BPV-1 DNA probe was able to detect both integrated and extrachromosomal sequences (Fig. 6B), which indicated that only viral sequences undergo excision from the transgene.

Analysis using the pBR322 DNA probe demonstrated that the integrated sequences were present at an approximately equivalent copy number in the DNAs of the tumors, the abnormal skin samples, and the internal organs (Fig. 6A). These data indicate that the integrated copies of the transgene had not undergone any significant major amplification or deletion in any of the tissues, including the tumors. Also, since no bands smaller than the ~17-kb band seen in the normal tissues were noted when the procaryotic probe was used in the samples with extrachromosomal BPV-1 DNA, excision of the viral genome would not appear to involve homologous recombination with deletion, at least within the BPV 1.69 repeated segment of the integrated viral transge-

TABLE 3. Stages of tumorigenesis in the skin of BPV 1.69 mice

Characteristic	Normal skin	Benign fibroproliferation		Fibrosarcoma
		Fibromatosis	Aggressive fibromatosis	
Tissue phenotype				
Histopathology	No abnormality	Dermal fibroblastic proliferation with atrophy of epidermis and dermal appendages	Dense fibroblastic proliferation with atrophy of epidermis and dermal appendages	Fibroblastic tumor with sheets of spindle-shaped cells and high mitotic index
Extrachromosomal BPV DNA	−	+	++	++
BPV transcription	−	+	++	++
Cell phenotype of dermal fibroblasts				
Culture morphology	Flat (large)	Flat	Flat (spindle shaped)	Focus forming (rounded)
Contact inhibited	+	+	±	−
Growth rate	Slow	Slow	Fast	Fast
Tumorigenic	−	−	+	+
BPV oncoproteins	−	?[a]	+	+

[a] Uncertain because of the low sensitivity of the assay used. Although the oncoproteins were not detected, low levels of viral RNA were present, which suggested that low levels of E5 and E6 were probably also present.

nome. In the 007 tumor, additional DNA bands were noted with the BPV-1 probe (Fig. 6B), which indicated that rearrangements in the extrachromosomal viral genomes had occurred. However, rearrangements of extrachromosomal DNA were not a general characteristic of tumor DNAs and, in addition, were noted in an occasional fibromatosis DNA. In the tumor tissues, we estimate the copy number of extrachromosomal DNA to be approximately 25 to 100 per cell (Fig. 6B). The abnormal skin from aggressive fibromatosis also carried high copy numbers of extrachromosomal viral sequences, equivalent to those detected in tumor cells. Therefore, it is not possible to distinguish protuberant tumors and aggressive fibromatosis on the basis of the state or copy number of the BPV-1 DNA in the tissues. However, skin from the nonaggressive fibromatosis contained only 3 to 10 extrachromosomal copies per cell (Fig. 6B). Therefore, the abnormal skin conditions, which were separated into two categories on the basis of pathological differences, could similarly be identified by the degree of amplification of extrachromosomal BPV-1 sequences. This distinction reflects not merely the proportion of cells containing extrachromosomal DNA in the sample but also the levels of DNA in the cells, since analysis of cultures of the fibroblasts from these tissues revealed the same levels of DNA (data not shown).

In all internal organs analyzed, the viral sequences were integrated and the organs appeared to be histologically normal. In one mouse with a protuberant fibrosarcoma, a lymphoma with involvement of the liver and spleen was also diagnosed. Analysis of the BPV-1 DNA in these organs revealed that the viral sequences had remained integrated, with no evidence of amplification. This finding indicated that the lymphoma was not likely a consequence of BPV-1 expression in this mouse, since the lymphoma had no detectable levels of BPV-1 RNA. This result demonstrates further that rapid proliferation of cells in BPV 1.69 transgenic mice is not, by itself, sufficient to induce excision or expression of BPV-1 DNA.

DISCUSSION

The BPV 1.69 line of transgenic mice affords a system with unique characteristics for the study of carcinogenesis in vivo. Specific abnormal conditions can be distinguished in the multistep progression to tumorigenesis in these mice. The transgenome exists latently integrated in the germ line of the mice and is transcriptionally inactive in all tissues until the development of abnormal skin and dermal tumors at 8 to 9 months of age. The abnormal skin is characterized by a fibromatosis consisting of benign proliferation of dermal fibroblasts. The protuberant tumors are histologically described as malignant fibrosarcomas. These are clearly distinct abnormalities which correspond to specific stages of tumorigenesis, as each can be defined by histopathology and by cellular characteristics. Cell cultures established from these lesions have biological properties that approximate those of the lesions from which they were derived. Being skin lesions, the abnormalities themselves are readily identifiable, even at the early stages, and therefore can be easily followed in vivo. The distinct stages of tumorigenesis observed in the dermal fibroblastic lesions of these mice have cell-heritable characteristics, both in vivo and in cell culture, and therefore afford an opportunity to dissect the various stages of tumorigenic progression. These distinct stages, each with their characteristic features, are discussed below, summarized in Table 3, and illustrated in Fig. 7.

Latency. Neither extrachromosomal BPV-1 DNA nor viral gene expression could be detected either in tissue samples or in cell cultures derived from normal skin. The BPV-1 transgenome, therefore, exists in a latent form integrated into a chromosome of these mice. Activation of viral gene expression correlates with the presence of extrachromosomal viral DNA in the cells. There is considerable tissue specificity with regard to activation of the viral genome in that it is limited to the skin and, in particular, to the dermal fibroblasts of the skin. The analyses in this paper have not revealed the nature of the initiating event(s) which results in transcriptional activation of the viral genome and in the excision and amplification of the viral DNA. It is of interest, however, that there is a predilection for tumor formation at sites of wounding and irritation, which suggests that extracellular factors may be involved in the initiation of these events. The accessibility of normal skin containing integrated copies of the viral genome, in concert with cell cultures derived from that normal skin, provides a format for identifying the intrinsic and extrinsic conditions and factors

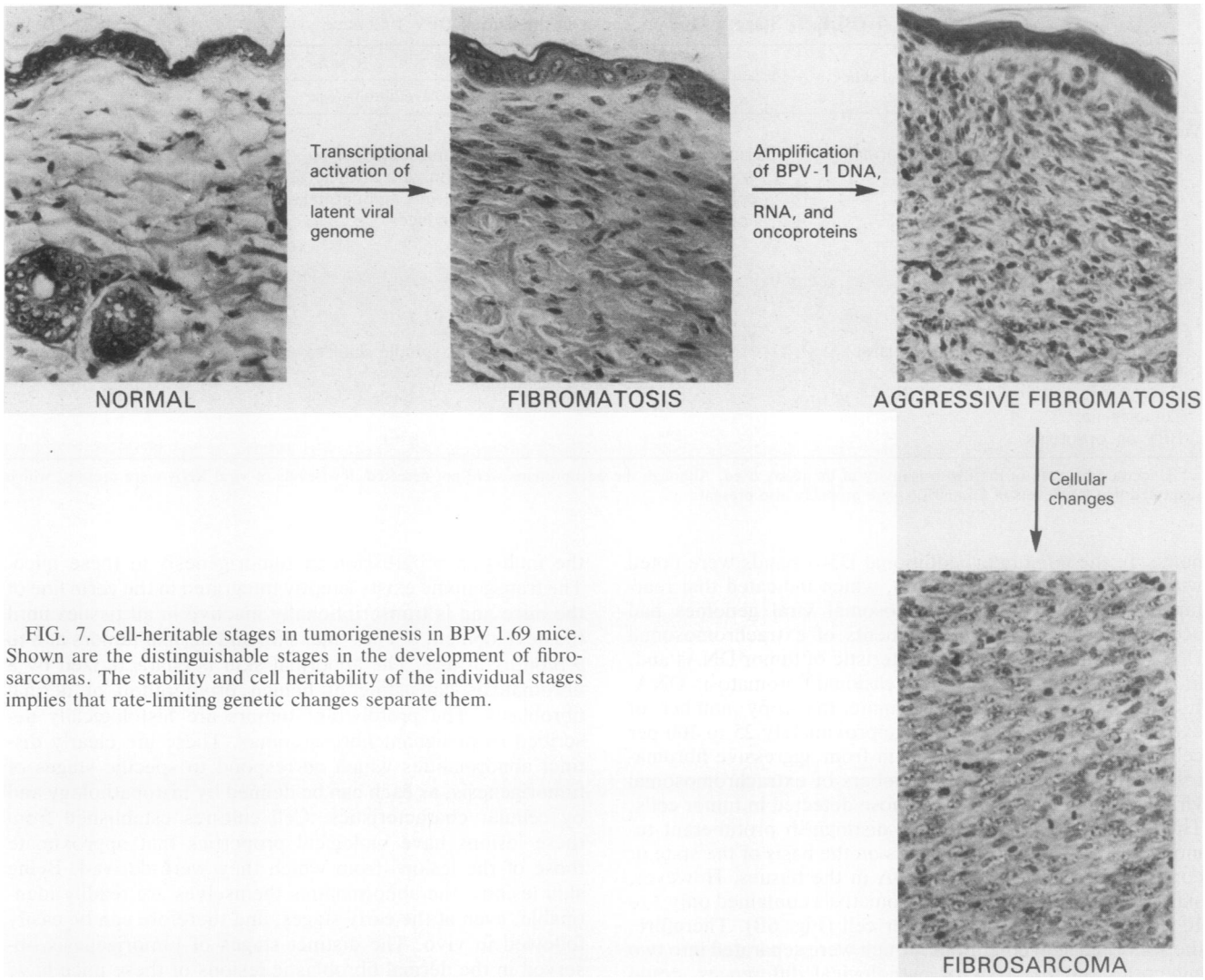

FIG. 7. Cell-heritable stages in tumorigenesis in BPV 1.69 mice. Shown are the distinguishable stages in the development of fibrosarcomas. The stability and cell heritability of the individual stages implies that rate-limiting genetic changes separate them.

which are necessary for the induction of viral gene expression.

Proliferation. The abnormal skin initially described by Lacey et al. (15) is characterized by a proliferation of dermal fibroblasts. The abnormal appearance of the skin results from a combination of thickening of the dermis and atrophy of skin appendages. The fibromatoses have been divided into a mild form and an aggressive form, depending on the extent of dermal fibroblastic proliferation (Fig. 7). Cell cultures can be established from lesions representative of the different abnormal conditions involving the dermal fibroblasts. These analyses have shown that cell cultures derived from fibromatoses and from aggressive fibromatoses show cell-heritable phenotypes which can be stably maintained in culture. The cells cultured from the lesions of the mild form of fibromatosis and from the lesions of aggressive fibromatosis have different growth characteristics. Cells from the nonaggressive form of fibromatosis lesions grow poorly in culture, do not grow under low-serum conditions, and are not tumorigenic in either syngeneic or nude mice. In contrast, cells from the aggressive fibromatosis lesions grow rapidly, with cell doubling times similar to those of the tumor cell lines. These cells are able to grow under low-serum conditions and are tumorigenic in syngeneic and nude mice. Despite their tumorigenicity, cells derived from the aggressive fibromatosis lesions do not form the dome-shaped foci characteristic of the tumor cell lines in culture. Furthermore, the mild and aggressive fibromatoses can be distinguished by the levels of viral DNA, of viral transcription, and of expression of the viral oncoproteins. The aggressive fibromatoses contain higher copy numbers of viral DNA than do the mild fibromatoses. The levels of RNA and viral oncoproteins parallel the DNA levels. Thus, the proliferative capacity of the benign fibromatoses cells appears to correlate directly with the levels of viral gene expression and with the levels of viral oncoproteins. The observation that the mild form of fibromatosis is stable and does not rapidly progress to aggressive fibromatosis in vitro implies that there may be restrictions on that progression which are not immediately overcome under conditions of controlled viral gene expression in cell culture.

Neoplasia. The neoplasias which arise reproducibly in BPV 1.69 mice are exclusively fibrosarcomas of the dermis. The cell lines established from these tumors have a phenotype which mirrors the biology of the lesions from which they were derived. The tumor cells are tumorigenic in both syngeneic and nude mice, grow well under low serum conditions, and form dome-shaped foci in tissue culture. These characteristics are stable in culture. It is somewhat surprising that the culture characteristics of cells derived from the fibrosarcomas are quite similar to those of cells derived from the aggressive fibromatosis lesions despite the clear in vivo distinctions between these lesions. Indeed, the

only in vitro characteristic which distinguishes these lesions is the ability of the cells derived from the tumors to form the dome-shaped foci of non-contact-inhibited cells (Fig. 1D). The tumor tissues and their derived cell lines contain a high copy number of the viral DNA, and the viral genome is transcriptionally active. In general, the levels of viral DNA and the levels of viral transcription for these tumors are similar to those seen for the aggressive fibromatosis cells in vivo and in vitro. In addition, the levels of the E5 oncoprotein are similar between the aggressive fibromatoses and the fibrosarcomas. In at least one case (tumor 851 [Fig. 4, lane c, and Fig. 5, lane k]), a high level of E5 protein was expressed despite a relatively low level of viral gene expression. Thus, although the proliferative capacity of the fibromatoses correlated well with the levels of oncoprotein expression, this was not necessarily the case with the tumors. This finding suggests that the levels of the viral oncoproteins themselves cannot explain all of the steps in the multistep tumor progression seen within these mice and therefore implicates additional changes in the later stages of tumor formation. We conclude, therefore, that oncogenesis in BPV 1.69 mice must involve a distinct event or set of events, some of which are not a direct consequence of BPV-1 gene expression. Elevated levels of the BPV-1 oncoproteins are, by themselves, apparently insufficient for progression to malignancy. The data would indicate, however, that expression of the BPV-1 oncoproteins is necessary as an initial step in tumor formation and that additional nonviral events must be implicated in this multistep tumor formation. Evidence in support of this hypothesis comes from the recent karyotypic analysis carried out by Valerie Lindgren on the various pathologic abnormalities seen within these mice (V. Lindgren et al., submitted for publication). The fibromatoses are essentially diploid, and the aggressive fibromatoses, which are aneuploid, have no characteristic chromosomal abnormalities. In contrast, specific chromosomal abnormalities characterize the fibrosarcomas that develop in BPV 1.69 mice.

Novel model for studying multistep tumorigenesis. These studies have shown that the BPV 1.69 transgenic mouse system provides a unique system for studying multistep carcinogenesis. There are three clearly defined stages: (i) latency, (ii) benign proliferation, and (iii) malignant progression. Within these stages, there are probably substages. In particular, the benign proliferation stage appears to have two stable substages, the mild and aggressive forms of fibromatosis (Fig. 7 and Table 3). Furthermore, since the pathology involves the skin, it can readily be assessed in vivo in animals which reproducibly recapitulate the onset and progress of the different tumorigenic stages. This presents a unique system for identifying factors that can affect this progression and ultimately for studying the molecular events that mediate tumor progression.

The stages in tumorigenesis described here may also be relevant for understanding the biology of papillomaviruses, which are now recognized to be associated with a variety of naturally occurring cancers. In animals, papillomavirus-induced tumorigenesis has been best studied for the Shope papillomavirus in skin lesions of rabbits (20, 23) and BPV-4 in alimentary tract carcinomas in cattle (12). In humans, papillomaviruses have been principally associated with cutaneous carcinomas in patients with epidermodysplasia verruciformis (18) and, more recently, in anogenital carcinomas, most notably cervical carcinoma (26). In each of these associations, carcinogenic progression occurs from a virally included benign lesion. The benign proliferative lesions have active expression of the viral gene products similar to expression of the benign proliferative lesions within transgenic mice. Tumor progression associated with the papillomaviruses in nature also appears to involve additional cellular genetic events. Therefore, tumor progression in BPV 1.69 mice may exemplify some of the stages of tumor progression seen naturally with the papillomaviruses. The ability to identify distinct stages in tumor progression and to establish stable cell lines representative of each stage will allow this process to be studied at molecular and biochemical levels.

ACKNOWLEDGMENTS

We are grateful to Carl Baker and Jacek Skowronski for critical reviews of the manuscript. We are grateful to Carol Comlish for preparation of the manuscript.

This study was supported in part by intramural funds of the Laboratory of Tumor Virus Biology, National Cancer Institute, and in part by grants from Monsanto Co., The American Cancer Society, and the National Cancer Institute to D.H.

LITERATURE CITED

1. **Androphy, E. J., J. T. Schiller, and D. R. Lowy.** 1985. Identification of the protein encoded by the E6 transforming gene of bovine papillomavirus. Science **230**:442–445.
2. **Baker, C. C., and P. M. Howley.** 1987. Differential promoter utilization by the bovine papillomavirus in transformed cells and in productively infected wart tissues. EMBO J. **6**:1027–1035.
3. **Bishop, J. M.** 1987. The molecular genetics of cancer. Science **235**:305–311.
4. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
5. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**:1991–1995.
6. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:266–267.
7. **Foulds, L.** 1954. The experimental study of tumor progression: a review. Cancer Res. **14**:327–339.
8. **Gross-Bellard, M., P. Oudet, and P. Chambon.** 1973. Isolation of high molecular weight DNA from mammalian cells. Eur. J. Biochem. **36**:32–38.
9. **Hanahan, D.** 1986. Oncogenesis in transgenic mice, p. 349–363. *In* T. Graf and P. Kahn (ed.), Oncogenes and growth control. Springer-Verlag KG, Berlin.
10. **Howley, P. M.** 1987. The role of papillomaviruses in human cancer, p. 55–73. *In* V. T. DeVita, S. Hellman, and S. A. Rosenberg (ed.), Important advances in oncology—1987. Lippincott, Philadelphia.
11. **Howley, P. M., and R. Schlegel.** 1987. Papillomavirus transformation, p. 141–166. *In* N. P. Salzman and P. M. Howley (ed.), The Papovaviridae 2: the papillomaviruses. Plenum Publishing Corp., New York.
12. **Jarrett, W. F. H., P. E. McNeil, W. I. R. Grimshaw, I. E. Selman, and W. I. M. McIntyre.** 1978. High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papillomavirus. Nature (London) **274**:215–217.
13. **Klein, G.** 1987. The approaching era of the tumor suppressor genes. Science **238**:1539–1545.
14. **Knudson, A. G.** 1986. Genetics of human cancer. Annu. Rev. Genet. **20**:231–251.
15. **Lacey, M., S. Alpert, and D. Hanahan.** 1986. The bovine papillomavirus virus genome elicits skin tumors in transgenic mice. Nature (London) **322**:609–612.
16. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical re-examination. Biochemistry **16**:4763–4751.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

18. **Orth, G.** 1987. Epidermodysplasia verruciformis, p. 199–244. *In* N. P. Salzman and P. M. Howley (ed.), The Papovaviridae 2: the Papillomaviruses. Plenum Publishing Corp., New York.
19. **Palmiter, R. D., and R. L. Brinster.** 1986. Germ line transformation of mice. Annu. Rev. Genet. **20**:465–499.
20. **Rous, P., and J. W. Beard.** 1935. The progression of carcinoma of virus-induced rabbit papillomas (Shope). J. Exp. Med. **62**: 523–548.
21. **Schiller, J. T., W. C. Vass, and D. R. Lowy.** 1984. Identification of a second transforming region of bovine papillomaviruses. Proc. Natl. Acad. Sci. USA **81**:7880–7884.
22. **Schlegel, R., M. Wade-Glass, M. S. Rabson, and Y. C. Yang.** 1986. The E5 transforming gene of bovine papillomavirus encodes a small, hydrophobic polypeptide. Science **233**:464–467.
23. **Shope, R. E.** 1933. Infectious papillomatosis of rabbits. J. Exp. Med. **58**:607–624.
24. **Stenlund, A., J. Zabielski, H. Ahola, J. Moreno-Lopez, and U. Pettersson.** 1985. Messenger RNAs from the transforming region of bovine papilloma virus type 1. J. Mol. Biol. **182**:541–554.
25. **Yang, Y.-C., H. Okayama, and P. M. Howley.** 1985. Bovine papillomavirus contains multiple transforming genes. Proc. Natl. Acad. Sci. USA **82**:1030–1034.
26. **zur Hausen, H., and A. Schneider.** 1987. The role of papillomaviruses in human anogenital cancer, p. 245–264. *In* N. P. Salzman and P. M. Howley (ed.), The Papovaviridae 2: The papillomaviruses. Plenum Publishing Corp., New York.