

## Human Adenovirus Type 12

### *Crossing Species Barriers to Immortalize the Viral Genome*

Walter Doerfler

#### Summary

When viruses cross species barriers, they often change their biological and pathogenetic properties. In the author's laboratory the nonproductive interaction of Syrian hamster cells with human adenovirus type 12 (Ad12) has been studied. Ad12 induces undifferentiated tumors in newborn hamsters (*Mesocricetus auratus*) at high frequency. Ad12 inefficiently enters hamster (BHK21) cells, and only small amounts of viral DNA reach the nucleus. Viral DNA replication and late transcription are blocked. In Ad12-induced tumor cells, multiple copies of viral DNA are chromosomally integrated. The integrated viral DNA becomes *de novo* methylated. Cellular DNA methylation and transcription patterns in Ad12-transformed cells and in Ad12-induced tumor cells are altered. These changes may be related to the oncogenic potential of Ad12 in hamsters. In this chapter, concepts and techniques for the study of the Ad12-hamster cell system are summarized.

**Key Words:** Abortive adenovirus type 12 infection; crossing species barriers; Ad12-induced hamster tumors; Ad12 DNA integration; *de novo* methylation of integrated foreign (Ad12) DNA; alterations of cellular DNA methylation patterns.

#### 1. Introduction

Viruses have evolved as biological elements with a highly select coordination to specific hosts and/or host cells. There are numerous steps in the interaction between a virus and its host that have to be optimized for the virus to be capable of undergoing a fully productive replicative cycle in the most permissive cell system. These parameters range from the attachment of the viral particle on the cell surface to the mechanisms of release of the newly assembled virions from the infected cell or organism. This adaptive process involves a large number of different viral and cellular proteins, whose optimized activi-

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ties and interactions are required to proceed at a unique temporal schedule during the viral replication program. Any deviation from this—in evolutionary terms—presently normalized standard invariably will entail the loss of or decrease in viral reproduction.

There exists, however, a corollary in the philosophy of viral strategies. “Smart viruses” are those that abstain from killing the host cell, admittedly at the expense of maximal viral output, but at the gain of some other, usually long-term, advantage for this particular virus in an environment that has developed antiviral strategies. There are numerous ways for viruses to achieve the goal of long-term survival, if only for immortalizing their genomes and to scheme for better times or circumstances for the rescue of the informational essence of the virion.

A not infrequently chosen contingency resides in taking refuge in a different host, although the virus initially cannot anticipate whether such untested territory will be amenable to and prove useful for viral survival. In its endeavor to survive, however, the viral parasite will breach a crucial law of survival—the respect for host specificity—and may cross species barriers, thus attempting the “unspeakable” in order to gain unprecedented, if unpredictable, advantages.

In many instances, the outcome of such viral experimentation will be frustrated. Nevertheless, the large number of virions usually produced in productive infections allows for many failures during risky adventures into unproven territories. The survivors of the successful events in these interactions are viruses that we encounter as “evolving viruses” with quite uncharacteristic and unforeseeable properties, which can cause catastrophic problems in medical virology. Many of the truly lethal viruses of present medical practice have evolved through such strategies of still poorly understood switches in their host ranges. Among others, viruses that presently raise serious concerns in the medical community include HIV 1 and 2, coronavirus severe acute respiratory syndrome, Marburg virus, and avian influenza virus H5N1 (1,2). These fatal disease-causing agents have alerted virologists to the potential of viruses that succeed in crossing species barriers and, in the course, acquire novel properties.

There are representatives of this class of viruses that have less immediate medical relevance but, nevertheless, have significance as models to study the essentials of nonproductive virus–host transitions. In my laboratory we have studied human adenovirus (Ad) type 12 and its interaction with cells from the Syrian hamster (*Mesocricetus auratus*) (3–5). Ad12 undergoes a fully productive cycle in human cells in culture but induces undifferentiated tumors in newborn Syrian hamsters (6–8). In crossing the barrier from human to hamster, Ad12 gains oncogenic potential. There is no evidence that human Ads are involved in human tumorigenesis (9–12). When Ad12 infects human cells, all infected cells are killed in the course of this productive interaction. Hence, there are no surviving cells that could be transformed into tumor cells. Therefore, it is not

surprising that Ad12 has not been identified as oncogenic in humans. It cannot be ruled out that Ad12 could be involved, however rarely, in a hit-and-run mechanism in human oncogenesis. This possibility becomes more plausible when one considers that, among hamster tumor cells, revertant cells have been isolated that have lost all of the previously integrated Ad12 genomes but have retained their oncogenic potential. Ad12 infection of hamster cells in culture is completely abortive; the replication block lies before viral DNA replication. The infected cells continue to replicate and show no cytopathic effect. Thus, in rare instances Ad12-infected hamster cells can go on to develop into tumor cells and form tumors in animals.

Among the factors contributing to the oncogenic potential of Ad12 in Syrian hamsters are the following:

1. The abortive mode of infection and the survival and continued growth of Ad12-infected hamster cells (3).
2. The insertion of Ad12 DNA into the host genome (13,14).
3. Alterations of cellular DNA methylation and transcription patterns (15–17).
4. The Ad12 E1 and E4 gene products (reviewed in ref. 18).
5. Downregulation of cellular major histocompatibility complex genes by Ad12 (19,20).
6. Downregulation of cellular defense genes by Ad12 (20).
7. Many additional unknown factors.

In the first, major, part of this chapter, concepts for the study of the abortive Ad12–hamster cell system and of the tumor production by Ad12 in newborn hamsters will be described. The second part will present a summary of the techniques employed in these studies.

### **1.1. Main Features of the Abortive Ad12-Hamster Cell System**

Primary hamster cells or the baby hamster kidney (BHK21) cell line are nonpermissive for infection with Ad12; the infection is abortive. However, the same cells are permissive for infection with Ad2, and this infection is productive (3,21). The following steps in the virus–host interaction have been characterized:

1. Ad12 adsorbs to the hamster cell surface, although much less efficiently than to human cells (22).
2. Ad12 particles enter the cytoplasm.
3. Ad12 DNA can be found in the nuclei of hamster cells (13,22).
4. Ad12 DNA associates with the chromosomes (23).
5. Ad12 DNA can integrate into the host chromosome (13,24–26).
6. Some of the early Ad12 functions are transcribed, although inefficiently (22,27).
7. By DNA array analyses, a limited number of changes in cellular transcription patterns can be documented upon the infection of BHK21 cells with Ad12 (Dorn, A. and Doerfler, W. unpublished results).
8. Ad12 DNA replication cannot be detected (3,22,28).

9. Ad12 VA and L1 RNAs are not transcribed (29).
10. The region downstream of the major late promoter (MLP) of Ad12 DNA carries a 33-bp-long mitigator element,\* which inactivates the MLP in hamster cells (30).
11. Late Ad12 genes are not detectably transcribed (28,31).
12. *Per definitionem*, new Ad12 virions are not produced in the abortive system (3).
13. There is no cytopathic effect in Ad12-infected hamster cells (the infected cells continue to replicate [3]).
14. As one of the biological consequences of the abortive infection of hamster cells, Ad12 induces undifferentiated tumors in newborn hamsters. (Ad2 fails to do so. The abortive mode of infection enables the Ad12 genome to become permanently fixed in the host genome and to survive for many cell generations. Productive infections would kill all infected cells.)

## 1.2. Attempts to Complement the Defect in Ad12 Replication in Hamster Cells

In the search for factors missing in hamster cells that would sustain Ad12 replication, we have tried to complement these defects in a stepwise manner. The nonpermissiveness of hamster cells for Ad12 replication seemed to be restricted to this particular serotype (since Ad2 underwent productive replication in hamster cells). Hence, there had to be a highly specialized barrier or barriers preventing Ad12 replication in hamster cells.

### 1.2.1. Ad12 in BHK21 Hamster Cells

BHK297-C131 hamster cells (32) constitutively express the E1 region of the integrated left 18.6% fragment of the Ad5 genome (31,33). The characterization of these cells is as follows:

1. Ad12 DNA can replicate to a limited extent.
2. Ad12 late transcripts are synthesized.
3. Ad12 fiber mRNA is transcribed, and has the correct nucleotide sequence, tripartite leader and polyA tail.
4. However, fiber protein is not produced (raising the possibility of an additional translational block in this Ad5-E1-complemented hamster cell system).
5. Ad12 virions are not made.

We conclude that the availability of apparently sufficient concentrations of the Ad5 E1 functions facilitates the activation of Ad12 replication and transcription machinery. Although the late fiber mRNA seems to have most, if not all, properties of *bona fide* Ad12 fiber mRNA, it cannot be translated into fiber protein.

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\*This mitigator is not found in the genome of Ad2. Excision of the mitigator element from the Ad12 genome renders the MLP active in hamster cells and increases its activity in human cells.

### 1.2.2. Overexpression of the Ad12 pTp or E1A Genes or of the Ad2 pTP or E1A Genes: A New Role for pTP in Early Ad12 Infection?

The cloned pTP or E1A gene under the control of the human cytomegalovirus (HCMV) promoter was transfected into BHK21 cells, which were subsequently infected with Ad12. The results were (34): (1) Ad12 DNA can replicate in these cells, although less efficiently than in permissive human cells; and (2) Ad12 late transcripts are not readily detectable (traces might be found late after infection). Although this approach was not particularly revealing for understanding the nature of the immediate block for Ad12 replication in BHK21 hamster cells, it yielded the unexpected result that overexpression of the Ad12 pTP gene alone, without enhanced transcription of the *E1A* genes, led to Ad12 DNA replication. It is conceivable that above-threshold concentrations of Ad12 pTP were capable of exerting previously unknown functions in preparing the hamster cells for nuclear import and replication of Ad12 DNA (even in the absence of large amounts of *E1A* gene products and beyond the well-known role that pTP can play in the initiation of Ad DNA replication). The data may suggest a novel role of Ad12 pTP in supporting Ad12 DNA nuclear import and replication in a nonpermissive cellular environment. What additional functions might pTP exert in controlling the uptake and transport of Ad12 DNA? Does the Ad12 DNA-bound TP in the infecting virion play a similar role?

### 1.2.3. Overexpression of the Human Coxsackie Ad Receptor in Hamster Cells

Following transfection, the human Coxsackie Ad receptor gene was transiently overexpressed in BHK21 hamster cells, which were subsequently infected with Ad12 at 8 h after transfection (22). The results were: (1) these cells develop a cytopathic effect at approx 24 h p.i.; and (2) the transport and uptake of Ad12 DNA into the hamster cell nuclei is markedly enhanced and even more efficient than in Ad2-infected BHK21 cells.

In a previous study on Ad12 infection of human embryonic kidney cells, we demonstrated that the extracellular virions, which were isolated from the medium, had a 10-fold higher specific infectivity per virus particle than the intracellular virions (35). By the then-available technology, gross physical differences could not be documented in the viral DNA and protein moieties between the intra- and extracellular Ad12 particles. It is conceivable that antecedent and/or during the release of the Ad12 virions from the cell, subtle alterations, perhaps the result of folding or processing modifications of virion proteins, bestow enhanced infectivity on the extracellular virions.

### 1.3. Synopsis of the Ad12 Hamster Tumor System

Ad12 can transform hamster cells in culture at extremely low efficiency. However, after injection into newborn Syrian hamsters (*Mesocricetus auratus*), tumors develop at high frequency (6–8,36). Recently, we have reported on the consequences of intramuscular injection of Ad12 and the intraperitoneal dissemination of multiple tumors within about 30 d of virus application in 70–90% of the animals surviving injection (8). Individual intraperitoneal tumors are of varying sizes and can exhibit identical or different integration profiles for the Ad12 genomes. The latter tumors are most likely of different origins; the former could be seed metastases within the animal's peritoneal cavity. Integration patterns of persisting Ad12 genomes have been determined by restriction cleavage of total intranuclear tumor cell DNA followed by Southern blotting and hybridization to intact Ad12 DNA or to the terminal fragments of the viral DNA (for methods, see refs. 8,24,25,37). Detailed analyses on more than 100 different tumors have documented the clonal derivation of Ad12-induced hamster tumors (37).

#### 1.3.1. On Ad Type 12 Oncogenesis

All tumor cells carry multiple copies of integrated, but no free, Ad12 DNA. Ad12 DNA usually inserts at only one chromosomal site. In rare instances (1/60), two insertion sites of Ad12 DNA in the hamster tumor genome have been observed (37). The sites of viral DNA integration are different from tumor to tumor, except for tumors derived as intraperitoneal seed metastases. Ad12-induced tumors are clonal. In all cells derived from one tumor, integration sites are identical. In the Ad12-induced hamster tumor cells, only some of the early Ad12 genes are transcribed at low efficiency. Viral transcription profiles are very similar from tumor to tumor (8). Late genes might be transcribed at extremely low levels, if at all. DNA array analyses of Ad12-induced tumor cells reveal both similarities and differences in cellular transcription patterns among different tumors (8). In an Ad12-induced hamster tumor cell line, about 1 kb of the adjacent cellular DNA showed changes in its methylation profile. Apparently, the insertion of foreign DNA into an established mammalian genome can alter DNA methylation at the site of foreign DNA insertion (38). In addition, foreign DNA insertion into an established genome can elicit alterations in DNA methylation and transcription patterns not only at the site of foreign DNA insertion but also at loci remote from the integration site (15–17).

### 1.3.2. Consequences of Foreign DNA Insertion Into Established Mammalian Genomes

#### 1.3.2.1. *DE NOVO* METHYLATION OF INTEGRATED FOREIGN DNA

When foreign DNA, such as Ad12 DNA in hamster cells, is genomically integrated into a mammalian genome, it frequently becomes *de novo* methylated (24,39) by an unknown mechanism. DNA methyltransferases 1 and 3a plus 3b seem to cooperate in this enzymatic reaction. The criteria for specific 5'-CG-3' dinucleotide selection in the methylation reaction are still unknown. *De novo* methylation is also important in embryonic development: patterns of DNA methylation are erased and subsequently *de novo* re-established. Because promoter methylation serves as a long-term signal for gene inactivation (40), the introduction of 5-mC residues at specific sites has been considered an ancient defense against the activity of foreign genes (41,42). The *de novo* methylation reaction of DNA has more generally important functions in many biological systems that are not directly related to virology. These include the following:

1. Gene inactivation in transgenic cells and organisms.
2. Genetic imprinting.
3. X-chromosome inactivation.
4. Altered transcription patterns during embryonal development.
5. Long-term silencing of retrotransposons.
6. Changes in transcription patterns in tumor cells.
7. Cloning of organisms.
8. Gene therapy.

#### 1.3.2.2. INDIRECT, FAR-REACHING INSERTIONAL MUTAGENESIS AT SITES REMOTE FROM THE INTEGRATION LOCUS

The insertion of foreign (Ad12, lambda, or plasmid) DNA into established mammalian genomes can be associated with extensive alterations in DNA methylation patterns in repetitive DNA sequences, e.g., the retrotransposon genomes of the intracisternal A particles (15). Changes in the methylation patterns of cellular genes can also be observed (major histocompatibility complex, IgC $\mu$ , serine protease, cytochrome P450) (15). Thus, the integration of foreign DNA (several hundred kb to 1 Mb) on one chromosome, as in Ad12-induced tumor cells, might lead to structural perturbations of chromatin. This perturbation is hypothesized to be transmitted to neighboring chromosomes in interphase nuclei, possibly by interactions via the nuclear matrix. Methylation and transcription patterns can be altered *in cis* and *in trans*. The author pursues the concept that these changes are critically involved in causing the oncogenic phenotype (43).



### *1.3.3. Loss of All Integrated Ad12 DNA Sequences From Ad12-Induced Hamster Tumor Cells Is Compatible With the Maintenance of the Oncogenic Phenotype*

When Ad12-induced tumor cells are passaged continuously in culture, spontaneous revertants of these tumor cells occasionally arise that exhibit altered (usually more fibroblastic) morphology and have lost all or next to all integrated viral DNA sequences. These revertants still retain their oncogenic potential when reinjected into hamsters (36,44). Hence, mechanisms other than those enacted by proteins encoded in the Ad12 E1 region must play a role at least in the maintenance of the Ad12-induced oncogenic phenotype. These findings have led me to study alterations in the methylation and transcription patterns of cellular genes and repetitive sequences in Ad12-transformed and Ad12-induced tumor cells as alternate important parameters in research on viral oncogenesis (43).

#### 1.3.3.1. CHARACTERISTICS OF THE REVERTANT TR12 DERIVED FROM THE AD12-TRANSFORMED BHK21 HAMSTER CELL LINE T637

The Ad12-transformed hamster cell line T637 was derived by infecting cells of the BHK21 hamster fibroblast line (45) in culture with Ad12 (46). Cell line T637 exhibits epitheloid morphology and differs distinctly from the parent fibroblastic BHK21 cells. Each T637 cell carries approx 15 copies of Ad12 DNA, which are integrated into the hamster cell genome (14,15,24,25) at a single chromosomal location in a “pearl necklace”-like array (23). Upon continuous passage of T637 cells in culture, morphological revertants with a more fibroblastic cell morphology arise. Several of these revertants, designated TR, have been cloned and characterized (47).

In the TR3 revertant of T637 cells, Ad12 DNA could no longer be detected in the cellular genome (15,47). In the revertant TR12, only one copy plus a 3.9-kb fragment from the right terminus of a second copy of Ad12 DNA still reside integrated (26), probably at the same chromosomal location (48), which harbors the 15 copies of Ad12 DNA in cell line T637. The 15 copies of Ad12 DNA in cell line T637 are less intensely methylated than the persisting Ad12 DNA in the revertant cell line TR12 (48). Detailed analyses of the patterns of methylation in the Ad12 integrates in cell line T637 and in its revertant TR12 are now being completed (26). It is conceivable that the extent of DNA methylation of transgenic DNAs, e.g., of the Ad12 genomes in cell lines T637 and TR12, is related to their stability of integration in the recipient genome. Could hypermethylation stabilize foreign DNA integrated into an established mammalian genome like the single copy of Ad12 DNA in cell line TR12?



## 2. Materials

1. 40 mM Hydroquinone.
2. Sodium bisulfite stock: prepare by dissolving 8.1 g of sodium bisulfite in 16 mL of degassed water by gently inverting the tube; 1 mL of 40 mM hydroquinone is finally added and the solution is adjusted to pH 5.0 by adding 0.6 mL of freshly prepared 10 M NaOH.
3. Glassmilk (Gene Clean II Kit Bio 101 Inc., Nista, CA).
4. Qiaquick gel extraction kit (Qiagen, Hilden, Germany).
5. *Escherichia coli* XL1BlueMRF' (Stratagene, La Jolla, CA).
6. Applied Biosystems 377 DNA sequencer.
7. GeneAmp 9600 system (Perkin Elmer, Norwalk, CT).
8. Taq DNA polymerase.
9. Avian myeloblastosis virus reverse transcriptase.
10. Tfl DNA polymerase (RT Access Kit, Promega, Madison, WI).
11. 2% Agarose gels.
12. pGEM-T vector (Promega, Madison, WI).
13. 1% Igepal CA-630 (Sigma).
14. Pefabloc SC protease inhibitor (Roche).
15. Branson B-12 sonifier.
16. Normal rabbit immunoglobulin G.
17. Protein A-agarose conjugate (Santa Cruz Biotechnology).
18. Ad12-pTP rabbit antiserum.
19. Proteinase K.
20. ss-M13-DNA purification kit (Qiagen, Hilden, Germany).
21. Positively charged nylon membranes (Roche).
22. Macherey/Nagel kit (Düren, Germany).
23.  $\alpha$ - or  $\gamma$ -<sup>32</sup>P-dCTP (3000 Ci/mmol).
24. Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany).
25. Sephadex G-50 columns (Roche).
26. DIG hybridization buffer (Roche).

## 3. Methods

This section will present an overview of the techniques used in our studies on the abortive interaction of Ad12 with hamster cells and on the analyses of Ad12-induced tumor cells. The details of some of these methods are described, unless they are well-known, routinely applied procedures used in molecular virology and biology. Among the more routine-based methods, which are described in detail in several of our referenced publications and will not be repeated here, are construction of expression vectors, expression clones, Southern blotting, analyses of newly synthesized viral/cellular DNA in transfected and infected cells, Western blot analysis, quantitative real-time reverse transcription-polymerase chain reaction (PCR) (22), preparation of protein extracts

(22,34), Ad12 tumor induction in newborn Syrian hamsters (8), DNA microarray technique: membranes, slides (8,20), determination of DNA methylation patterns by methylation-sensitive restriction endonucleases like the HpaII–MspI system, HhaI a.o. (15,24,48), arrays for the study of viral or cellular gene transcription (20), studies on viral DNA integration (8,14,24,25), and fluorescence *in situ* hybridization technique (23).

### 3.1. The Bisulfite Protocol of the Genomic Sequencing Method

1. Prepare genomic DNAs from Ad12-infected cells or from Ad12-induced hamster tumor cells following standard protocols (8,24).
2. The genomic DNA is then alkali-denatured in 0.3 M NaOH for 15 min at 37°C and for 3 min at 95°C.
3. Subsequently, treat the DNA with sodium bisulfite. Mix the denatured DNA solution (66  $\mu$ L) with 1.2 mL of the bisulfite solution (**Subheading 2., item 3**), overlay with mineral oil, and incubate at 55°C for 16 h in a water bath in the dark.
4. From the above reaction mixture (**step 3**), purify the DNA using glassmilk.
5. For desulfonation, add 3  $\mu$ L of 10 M NaOH and incubate for 10 min at 37°C.
6. The solution is thereupon neutralized, the DNA ethanol-precipitated, dried, and redissolved in 20  $\mu$ L H<sub>2</sub>O.
7. Amplify selected segments in the promoters by PCR with appropriate oligodeoxyribonucleotide (oligo) primers.
8. Clone reaction products into the pGEM–T vector (Promega, Madison, WI) after purification by using the Qiaquick gel extraction kit (Qiagen, Hilden, Germany) and transfect into *E. coli* XL1BlueMRF' (Stratagene, La Jolla, CA) applying standard procedures.
9. Isolate a number of clones and determine the nucleotide sequences with an Applied Biosystems 377 DNA sequencer.

The bisulfite reaction (49,50) converts all unmethylated C residues into U residues, which are copied during PCR amplification into T residues, whereas the 5-methyldeoxycytidine (5-mC) residues are refractory to the bisulfite conversion reaction. Thus, a C residue in the eventually determined nucleotide sequence proves the presence of a 5-mC residue in this position in the original genomic nucleotide sequence. All *bona fide* C residues score as Ts.

### 3.2. PCR Amplification

1. Perform PCR amplification in a reaction volume of 25  $\mu$ L in a GeneAmp 9600 system by using 250 ng of bisulfite-treated genomic DNA, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.7 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 1 U of Taq DNA polymerase, 0.1 mM of each of the four dNTPs, and 250 ng of each primer. Primer sequences are chosen based on the published Ad12 DNA sequence (51).
2. Amplification conditions are as follows: After the initial denaturation step at 94°C for 5 min, 35 cycles of denaturation at 94°C for 15 s, annealing for 15 s at tem-

peratures between 51°C and 57°C, and an extension reaction at 72°C for 30 s follows. A final extension step at 72°C for 5 min terminates the reaction.

### 3.3. Reverse Transcription-PCR

1. Reverse transcription reactions are performed in a one-step protocol, with 100–300 ng of DNase I-treated total RNA by using avian myeloblastosis virus reverse transcriptase and Tfl DNA polymerase following the manufacturer's guidelines.
2. The RT reaction is carried out at 48°C for 45 min, followed by PCR with 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min and elongation at 68°C for 2 min. Subsequently, a final elongation step at 68°C for 7 min is applied.
3. All reverse transcription (RT)-PCR reaction products are analyzed by electrophoresis on 2% agarose gels and are stained with ethidium bromide.
4. Products in the size range predicted from the published DNA sequence are purified from the gel, subcloned into the pGEM-T vector, and the DNA is sequenced.

### 3.4. Immunoprecipitation of the Ad12-pTP Complex

1. Transfect BHK21 cells with the cloned Ad12-pTP DNA by electroporation.
2. At 18 h after transfection, infect the cells with 100 plaque-forming units of Ad12 per cell.
3. At 28 h p.i., suspend about  $10^7$  cells in phosphate-buffered saline containing 1% Igepal CA-630, 0.1% sodium dodecyl sulfate (SDS), and 4 mM Pefabloc SC protease inhibitor.
4. The cells are disrupted by ultrasonic treatment for 2 min in a Branson B-12 sonifier. After removal of cell debris, treat cell extracts for 1 h at 4°C with 1.0 µg of normal rabbit immunoglobulin G and 20 µL of protein A-agarose conjugate.
5. Subsequently the beads are pelleted, and the lysate is incubated at 4°C overnight with 15 µL of Ad12-pTP rabbit antiserum and 30 µL of protein A-agarose.
6. Immunoprecipitates are collected by centrifugation for 5 min at 1000g and are washed four times with phosphate-buffered saline.
7. For the release of Ad12 DNA from this complex, suspend the beads in 50 µL of 10 mM Tris-HCl (pH 8.0), 2 mM ethylene diamine tetraacetic acid, 10 mM NaCl, 1% SDS, and treat for 3 h at 55°C with 1 µg of Proteinase K per mL.
8. This protocol is described in **ref. 34**. The DNA is then analyzed by standard restriction and blotting procedures.

### 3.5. Construction and Use of Single-Stranded DNA Arrays

1. Ad12-specific DNA segments corresponding to 28 different open reading frames of Ad12 DNA are amplified by PCR using appropriate primers and are cloned into M13mp18 vector DNA as described (8).
2. Prepare single-stranded (ss) M13-Ad12 DNAs (we used 28 different clones) using the ss-M13-DNA purification kit.
3. Denature the ssDNAs in 0.4 M NaOH, 5 mM ethylene diamine tetraacetic acid, fix on positively charged nylon membranes, and hybridize to <sup>32</sup>P-labeled cDNA.

(We used amounts of 20 or 100 ng of Ad12-M13 DNA for HeLa·Ad12 or BHK21·Ad12 DNA array analysis, respectively.)

4. For reverse transcription and  $^{32}\text{P}$ -labeling of cDNA, total RNA from mock- or Ad12-infected cells is isolated by using a Macherey/Nagel kit. In this reaction, 5  $\mu\text{g}$  of RNA, 2  $\mu\text{g}$  of oligo (dT) 12–18 primers, and 1 mM of each of the dNTPs are denatured at 65°C for 5 min, chilled on ice, then mixed with 5  $\mu\text{Ci}$  of ( $\gamma$ - $^{32}\text{P}$ )-dCTP (3000 Ci/mmol), 10 mM dithiothreitol, superscript II reaction buffer, and 300 U of superscript II reverse transcriptase and are incubated at 42°C for 1.5 h.
5. Subsequently, the  $^{32}\text{P}$ -labeled probes are purified by gel filtration on Sephadex G-50 columns and are hybridized to DNA arrays on membranes at 42°C for 20 h in DIG hybridization buffer.
6. Wash membranes three times for 20 min in 0.5X standard sodium citrate, 1% SDS at 65°C and expose for 14 h (HeLa·Ad12) or 48 h (BHK21·Ad12) to X-ray films.

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## References

1. Avian flu special. (2005) *Nature* **435**, 399–409.
2. The next pandemic? (2005) *Foreign Affairs* **84/4**, 3–64.
3. Doerfler, W. (1969) Nonproductive infection of baby hamster kidney cells (BHK21) with adenovirus type 12. *Virology* **38**, 587–606.
4. Doerfler, W. (1991) Abortive infection and malignant transformation by adenoviruses: Integration of viral DNA and control of viral gene expression by specific patterns of DNA methylation. *Adv. Virus Res.* **39**, 89–128.
5. Hösel, M., Webb, D., Schröer, J., and Doerfler, W. (2003) The abortive infection of Syrian hamster cells with human adenovirus type 12. *Curr. Top. Microbiol. Immunol.* **272**, 415–440.
6. Trentin, J. J., Yabe, Y., and Taylor, G. (1962) The quest for human cancer viruses. A new approach to an old problem reveals cancer induction by human adenoviruses. *Science* **137**, 835–841.
7. Huebner, R., Rowe, W. P., and Lane, T. W. (1962) Oncogenic effects in hamsters of human adenovirus types 12 and 18. *Proc. Natl. Acad. Sci. USA* **48**, 2051–2058.
8. Hohlweg, U., Hösel, M., Dorn, A., et al. (2003) Intraperitoneal dissemination of Ad12-induced undifferentiated neuroectodermal hamster tumors: de novo methylation and transcription patterns of integrated viral and of cellular genes. *Virus Res.* **98**, 45–56.

9. Green, M., Mackey, J. K., Wold, W. S. M., and Ridgen, P. (1977a) Analysis of human cancer DNA for DNA sequences of human adenovirus type 4. *J. Natl. Cancer Inst.* **62**, 23–26.
10. Green, M., Mackey, J. K., Wold, W. S. M., and Ridgen, P. (1977b) Analysis of human cancer DNA for DNA sequences of human adenovirus serotypes 3, 7, 11, 14, 16 and 21 in group B. *Cancer Res.* **39**, 3479–3484.
11. Green, M., Wold, W. S. M., Mackey, J. K., and Ridgen, P. (1977) Analysis of human tonsil and cancer DNAs and RNAs for DNA sequences of subgroup C (serotypes 1, 2, 5 and 6) human adenoviruses. *Proc. Natl. Acad. Sci. USA* **76**, 6606–6610.
12. Mende, Y., Schneider, P. M., Baldus, S. E., and Doerfler, W. (2004) PCR-screening of human esophageal and bronchial cancers reveals absence of adenoviral DNA sequences. *Virus Res.* **104**, 81–85.
13. Doerfler, W. (1968) The fate of the DNA of adenovirus type 12 in baby hamster kidney cells. *Proc. Natl. Acad. Sci. USA* **60**, 636–643.
14. Doerfler, W., Gahlmann, R., Stabel, S., et al. (1983) On the mechanism of recombination between adenoviral and cellular DNAs: the structure of junction sites. *Curr. Top. Microbiol. Immunol.* **109**, 193–228.
15. Heller, H., Kämmer, C., Wilgenbus, P., and Doerfler, W. (1995) Chromosomal insertion of foreign (adenovirus type 12, plasmid, or bacteriophage  $\lambda$ ) DNA is associated with enhanced methylation of cellular DNA segments. *Proc. Natl. Acad. Sci. USA* **92**, 5515–5519.
16. Remus, R., Kämmer, C., Heller, H., Schmitz, B., Schell, G., and Doerfler, W. (1999) Insertion of foreign DNA into an established mammalian genome can alter the methylation of cellular DNA sequences. *J. Virol.* **73**, 1010–1022.
17. Müller, K., Heller, H., and Doerfler, W. (2001) Foreign DNA integration. Genome-wide perturbations of methylation and transcription in the recipient genomes. *J. Biol. Chem.* **276**, 14,271–14,278.
18. Endter, C. and Dobner, T. (2004) Cell transformation by human adenoviruses. *Curr. Top. Microbiol. Immunol.* **273**, 163–214.
19. Zantema, A. and van der Eb, A. J. (1995) Modulation of gene expression by adenovirus transformation. *Curr. Top. Microbiol. Immunol.* **199/III**, 1–23.
20. Dorn, A., Zhao, H., Granberg, F., et al. (2005) Identification of specific cellular genes up-regulated late in adenovirus type 12 infection. *J. Virol.* **79**, 2404–2412.
21. Strohl, W. A., Rouse, H., Teets, K., and Schlesinger, R. W. (1970) The response of BHK21 cells to infection with type 12 adenovirus. 3. Transformation and restricted replication of superinfecting type 2 adenovirus. *Arch. Ges. Virusforsch.* **31**, 93–112.
22. Webb, D., Hösel, M., Hochstein, N., Dorn, A., Auerochs, S., and Doerfler, W. (2005) hCAR-enhanced entry of adenovirus type 12 (Ad12) into non-permissive hamster cells fails to elicit viral replication. In revision.
23. Schröer, J., Hölker, I., and Doerfler, W. (1997) Adenovirus type 12 DNA firmly associates with mammalian chromosomes early after virus infection or after DNA

- transfer by the addition of DNA to the cell culture medium. *J. Virol.* **71**, 7923–7932.
24. Sutter, D., Westphal, M., and Doerfler, W. (1978) Patterns of integration of viral DNA sequences in the genomes of adenovirus type 12-transformed hamster cells. *Cell* **14**, 569–585.
  25. Knoblauch, M., Schröer, J., Schmitz, B., and Doerfler, W. (1996) The structure of adenovirus type 12 DNA integration sites in the hamster cell genome. *J. Virol.* **70**, 3788–3796.
  26. Hochstein, N., Muiznieks, I., Mangel, L., Brondke, H., and Doerfler, W. (2006) Selection for transgenome stability and distinct hypermethylation: anatomy of a transgenome. Submitted.
  27. Ortin, J., Scheidtmann, K.-H., Greenberg, R., Westphal, M., and Doerfler, W. (1976) Transcription of the genome of adenovirus type 12. III. Maps of stable RNA from productively infected human cells and abortively infected and transformed hamster cells. *J. Virol.* **20**, 355–372.
  28. Schiedner, G. and Doerfler, W. (1996) Insufficient levels of NFIII and its low affinity for the origin of adenovirus type 12 (Ad12) DNA replication contribute to the abortive infection of BHK21 hamster cells by Ad12. *J. Virol.* **70**, 8003–8009.
  29. Jüttermann, R., Weyer, U., and Doerfler, W. (1989) Defect of adenovirus type 12 replication in hamster cells: Absence of transcription of viral virus-associated and L1 RNAs. *J. Virol.* **63**, 3535–3540.
  30. Zock, C. and Doerfler, W. (1990) A mitigator sequence in the downstream region of the major late promoter of adenovirus type 12 DNA. *EMBO J.* **9**, 1615–1623.
  31. Schiedner, G., Schmitz, B., and Doerfler, W. (1994) Late transcripts of adenovirus type 12 DNA are not translated in hamster cells expressing the E1 region of adenovirus type 5. *J. Virol.* **68**, 5476–5482.
  32. Visser, L., van Maarschalkerweerd, M. W., Rozijn, T. H., Wassenaar, A. D., Reemst, A. M., and Susenbach, J. S. (1980) Viral DNA sequences in adenovirus-transformed cells. *Cold Spring Harb. Symp. Quant. Biol.* **44**, 541–550.
  33. Klimkait, T. and Doerfler, W. (1985) Adenovirus types 2 and 5 functions elicit replication and late expression of adenovirus type 12 DNA in hamster cells. *J. Virol.* **55**, 466–474.
  34. Hösel, M., Webb, D., Schröer, J., Schmitz, B., and Doerfler, W. (2001) The over-expression of the adenovirus type 12 pTP or E1A gene facilitates Ad12 DNA replication in non-permissive BHK21 hamster cells. *J. Virol.* **75**, 16,041–16,053.
  35. Brüggemann, U., Klenk, H.-D., and Doerfler, W. (1985) Increased infectivity of extracellular adenovirus type 12. *J. Virol.* **55**, 117–125.
  36. Kuhlmann, I., Achten, S., Rudolph, R., and Doerfler, W. (1982) Tumor induction by human adenovirus type 12 in hamsters: loss of the viral genome from adenovirus type 12-induced tumor cells is compatible with tumor formation. *EMBO J.* **1**, 79–86.
  37. Hilger-Eversheim, K. and Doerfler, W. (1997) Clonal origin of adenovirus type 12-induced hamster tumors: nonspecific chromosomal integration sites of viral DNA. *Cancer Res.* **57**, 3001–3009.

38. Lichtenberg, U., Zock, C., and Doerfler, W. (1988) Integration of foreign DNA into mammalian genome can be associated with hypomethylation at site of insertion. *Virus Res.* **11**, 335–342.
39. Sutter, D. and Doerfler, W. (1980) Methylation of integrated adenovirus type 12 DNA sequences in transformed cells is inversely correlated with viral gene expression. *Proc. Natl. Acad. Sci. USA* **77**, 253–256.
40. Doerfler, W. (1983) DNA methylation and gene activity. *Ann. Rev. Biochem.* **52**, 93–124.
41. Doerfler, W. (1991) Patterns of DNA methylation—evolutionary vestiges of foreign DNA inactivation as a host defense mechanism—a proposal. *Biol. Chem. Hoppe-Seyler* **372**, 557–564.
42. Yoder, J. A., Walsh, C. P., and Bestor, T. H. (1997) Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* **13**, 335–340.
43. Doerfler, W. (2000) *Foreign DNA in Mammalian Systems*. Wiley-VCH, New York.
44. Pfeffer, A., Schubert, R., Orend, G., Hilger-Eversheim, K., and Doerfler, W. (1999) Integrated viral genomes can be lost from adenovirus type 12-induced hamster tumor cells in a clone-specific, multistep process with retention of the oncogenic phenotype. *Virus Res.* **59**, 113–127.
45. Stoker, M. and Macpherson, I. (1964) Syrian hamster fibroblast line BHK21 and its derivatives. *Nature* **203**, 1355–1357.
46. Strohl, W. A., Rabson, A. S., and Rouse, H. (1967) Adenovirus tumorigenesis: Role of the viral genome in determining tumor morphology. *Science* **156**, 1631–1633.
47. Groneberg, J., Sutter, D., Soboll, H., and Doerfler, W. (1978) Morphological revertants of adenovirus type 12-transformed hamster cells. *J. Gen. Virol.* **40**, 635–645.
48. Orend, G., Knoblauch, M., and Doerfler, W. (1995) Selective loss of unmethylated segments of integrated Ad12 genomes in revertants of the adenovirus type 12-transformed cell line T637. *Virus Res.* **38**, 261–267.
49. Frommer, M., McDonald, L. E., Millar, D. S., et al. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA* **89**, 1827–1831.
50. Zeschngk, M., Schmitz, B., Dittrich, B., Buiting, K., Horsthemke, B., and Doerfler, W. (1997) Imprinted segments in the human genome: different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. *Hum. Mol. Genet.* **6**, 387–395.
51. Sprengel, J., Schmitz, B., Heuss-Neitzel, D., Zock, C., and Doerfler, W. (1994) Nucleotide sequence of human adenovirus type 12 DNA: comparative functional analysis. *J. Virol.* **68**, 379–389.