Late Transcripts of Adenovirus Type 12 DNA Are Not Translated in Hamster Cells Expressing the E1 Region of Adenovirus Type 5

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Hamster cells are completely nonpermissive for the replication of human adenovirus type 12 (Ad12), whereas types 2 and 5 can replicate in hamster cells. The Ad5-transformed hamster cell line BHK297-C131, which carries the left terminal 18.7% of the Ad5 genome and expresses at least the viral E1A region, can somehow complement Ad12 DNA replication and the transcription of the late Ad12 genes. Since the interaction of Ad12 with hamster cells must constitute a significant factor in the induction of Ad12 tumors in neonatal hamsters, we have continued to examine details of this abortive virus infection. The late Ad12 mRNAs in BHK297-C131 cells are polyadenylated but are synthesized in reduced amounts compared with the Ad12 products in Ad12-infected human cells, which are permissive for viral replication. The late mRNA derived from the Ad12 fiber gene has been assessed for its structural properties. By cloning cDNA transcripts from this region and determining their nucleotide sequences, the authenticity of the complete Ad12 fiber sequence and the completeness of the Ad12-typical tripartite leader have been confirmed. Moreover, in Ad12-infected BHK297-C131 cells the Ad12 virus-associated RNA, a virus-encoded translational activator with the correct nucleotide sequence, is synthesized. Nevertheless, the synthesis of detectable amounts of Ad12 virion-specific proteins, and in particular that of the main viral antigens, hexons and fibers, cannot be documented. Cellular factors needed to promote late mRNA translation might be missing, or inhibitory factors might exist in Ad12-infected BHK297-C131 cells.

In a long-standing series of experiments, we have investigated the interaction of human adenovirus type 12 (Ad12) with BHK21 hamster cells (for reviews, see references 10 and 40), because Ad12 has been shown to induce tumors in hamsters (36). Ad12 infects BHK21 cells in culture, and the viral DNA reaches the nucleus and can be integrated into the cellular genome. Even with the most sensitive techniques, viral DNA replication cannot be detected. Early Ad12 genes are transcribed in hamster cells; the late genes, however, remain silent (21). The virus-associated (VA) RNA of Ad12 (13), a translational regulator, is not transcribed in hamster cells. The inactivity of the major late promoter of Ad12 DNA is likely responsible for the absence of late Ad12 DNA transcription in hamster cells. This inactivity is at least partly due to a 32-bp mitigator element in the downstream region of the Ad12 major late promoter (39-41). The cellular YY1 protein (28, 29) can interact with elements in the major late promoter of Ad12 DNA (39, 41).

Hamster cells are permissive for human Ad2 and Ad5. In hamster cells carrying the E1 region of Ad5, a complementing system, Ad12 DNA can replicate, and late genes can be transcribed, but Ad12 virions are not produced (15, 16). These findings raise the question of why the late Ad12-specific RNAs cannot be translated into the late virion structural proteins in the complemented system. Here, we show that in cells expressing the E1 region of Ad5 DNA, early and late Ad12 RNAs are synthesized, but Ad12 hexon and fiber protein cannot be detected by Western immunoblotting. The amounts of late Ad12 RNAs expressed in the complementing hamster cells are reduced in comparison with the amounts of late Ad12 RNAs transcribed in productively infected human cells. Ad12 VA RNA, which is required for efficient translation of viral mRNAs at late times after infection, as shown for Ad2 (25, 26, 35), is also synthesized in the complemented system. The Ad12-specific fiber RNA from the Ad5E1-complemented hamster cell system has been reverse transcribed into cDNA, the cDNAs have been cloned, and their nucleotide sequences have been determined. Most of these polyadenylated RNAs carry the authentic Ad12 fiber RNA sequence with the correct tripartite leaders attached. We conclude that the failure of late Ad12 RNAs to be translated contributes significantly to the nonpermissivity of hamster cells for Ad12 even in the presence of the complementing Ad5 E1 functions.

MATERIALS AND METHODS

Cell lines, virus, and virus infections. Human KB and HeLa cells, the baby hamster kidney cell line BHK21, and the Ad5-transformed hamster cell line BHK297-C131 (37) (referred to as C131 in Fig. 1 through 6) were grown in monolayer cultures in Dulbecco medium (4) supplemented with 10% fetal calf serum. Human Ad12 was replicated in KB suspension cultures and purified as described previously (9). For infection with Ad12, KB, BHK21, and BHK297-C131 cells were grown on monolayers to 40 to 50% confluence. KB cells were infected with CsCl-purified Ad12 at multiplicities of 20 to 25 PFU per cell. BHK21 and BHK297-C131 cells were infected with 80 to 100 PFU of Ad12 per cell.

Standard methods of molecular biology. Southern blotting (17, 31), isolation of cytoplasmic RNA (27), Northern (RNA) blotting (2, 19, 24), selection of polyadenylated RNA sequences (3), and oligolabeling of DNA (12) were performed by following standard protocols.

For the isolation of genomic DNA, standard procedures (33,
34) were adapted. KB and BHK297-C131 cells were grown on monolayers and infected with Ad12. At different times after infection, cells were washed with Tris-saline buffer and then treated with sodium dodecyl sulfate (SDS)-EDTA-proteinase K. Nucleic acids were extracted with phenol-chloroform and the DNA was precipitated with ethanol according to the standard protocols.

**Preparation of protein extracts from KB and BHK297-C131 cells.** Nuclear extracts were prepared according to published protocols (8, 39) from cells grown and infected on monolayers. As markers, Ad12 proteins from CsCl-purified Ad12 virions were lysed in 2× SDS loading buffer (100 mM Tris-Cl [pH 6.8], 20% [vol/vol] glycerol, 6% [wt/vol] SDS, 10% [vol/vol] β-mercaptoethanol, 0.01% [wt/vol] bromophenol blue).

**Protein gel electrophoresis and Western blotting.** Proteins were analyzed by electrophoresis in standard SDS–8% polyacrylamide gels (18). Commercial prestreained proteins were used (Sigma) as molecular weight size markers. After electrophoresis, the proteins were blotted onto nitrocellulose membranes (ECL-Hybrid; Amersham) by semidry blotting on Biometra FastBlot B33. After blocking unspecific binding by incubation in phosphate buffered saline (PBS) (11) containing 5% (wt/vol) milk powder and 0.1% (vol/vol) Tween 20, the membranes were incubated with an anti-Ad12 rabbit anti-serum diluted in PBS containing 5% (wt/vol) milk powder and 0.1% (vol/vol) Tween 20. After subsequent intensive washing in 0.1% (vol/vol) Tween 20 in PBS, an anti-rabbit antibody coupled with horseradish-peroxidase (Amersham) was applied. After further intensive washing, the proteins were stained by incubation in enhancer (ECL; Amersham) and subsequent exposure to an X-ray film for 20 to 60 s.

**cDNA synthesis and cloning.** All cDNAs were synthesized from poly(A)⁺ RNA isolated (3) 52 h postinfection with the ZAP cDNA synthesis system (Stratagene). The primer used for the synthesis of the first strand contained a poly(dT) region and a XhoI restriction site [5’-(GAGA), ACTAGTCTCAG (T)，13]. After the ligation of EcoRI adaptors to the blunt ends of the cDNAs and subsequent XhoI restriction, the cDNAs were size fractionated. The cDNA fragments larger than 500 bp were ligated to the Uni-ZapXR vector arms (Stratagene) and packaged with Gigapack II Gold packaging extracts (Stratagene) into λ phage heads. Recombinant phages were identified by plaque hybridization with the [32P]-labeled PstI-P fragment of Ad12 DNA. The cDNA fragments were subcloned by in vivo excision of pBluescript SK(−) from the λ vector Uni-ZapXR (30). The inserted DNA was analyzed by standard techniques including Southern blotting (17, 31) and nucleotide sequencing (23).

**Primer extension and PCR analyses.** DNase-treated cytoplasmic RNA (3 μg from infected and uninfected KB cells and 30 μg from infected and uninfected BHK297-C131 cells) was annealed to a 3’-VA RNA-specific 30-base oligodeoxyribonucleotide primer (5’-GGGATTCCTCTCCTGCTGTTGG GGTCCCT-3’) or a fiber RNA-specific 28-base oligodeoxyribonucleotide primer (5’-CTTACGATACGGTTGCTG GCCGTC-3’). After hybridization at 40°C for 16 h in 1 M NaCl-0.17 M HEPES (N-2-hydroxyethylpiperezine-N’-2-ethanesulfonic acid) (pH 7.5)-0.3 mM EDTA and subsequent ethanol precipitation, each of the primers was extended at 42°C for 90 min with 23 U of reverse transcriptase from Moloney murine leukemia virus (Stratagene) in the presence of 50 mM Tris-Cl (pH 8.3)-50 mM KCl-10 mM MgCl₂-1 mM dithiothreitol-1 mM EDTA-10 μg of bovine serum albumin. The cells were washed once with Tris-saline and treated with 10 μg of RNase, extracted with phenol-chloroform, and precipitated with ethanol.

For subsequent PCR analyses (22), different amounts of the VA-RNA-specific primer extension products were incubated with 100 ng of the 3’ VA-RNA-specific 30-base oligodeoxyribonucleotide primer plus 100 ng of a 5’ VA-RNA-specific 30-base oligodeoxyribonucleotide primer (5’-TTGAAATTCGTTGTTGGAAAGTAC-3’) in 50 mM KCl-10 mM Tris-Cl (pH 9.0)-0.1% (vol/vol) Triton X-100-1.5 mM MgCl₂-0.2 mM each deoxyribonucleoside triphosphate—2 U of Taq polymerase (Serva). The thermal cycling reaction was repeated 20 times in a Perkin-Elmer DNA Thermal Cycler 480 under the following conditions: 1 min at 92°C, 1 min at 64°C, and 1 min at 72°C. Identical amounts of the fiber-specific primer extension products were incubated with 100 ng of the fiber RNA-specific 28-base oligodeoxyribonucleotide primer plus 100 ng of a leader 1-specific 30-base oligodeoxyribonucleotide primer (5’-TTCTGCTCGTGGTTAACATGACCCCTG-3’) under the same conditions as described for VA-RNA PCR, with the exception that the thermal cycling reaction was repeated 25 times. Subsequently, DNA samples were purified by standard phenolchloroform (1:2) extraction, combined with gel loading buffer, and fractionated by electrophoresis on a 1.5% agarose gel or a 4% polyacrylamide gel. PCR products were visualized by ethidium bromide staining and subjected to UV photography. For the determination of the nucleotide sequence of the VA-RNA specific PCR products, the DNA was purified over spin columns (QIAquick-spin PCR purification kit; Qiagen) according to the manufacturer’s protocol.

**RESULTS AND DISCUSSION**

The complementation of deficient Ad12 functions in the Ad5-transformed hamster cell line BHK297-C131. Cell line C131 carries the left terminal 18.7% of the Ad5 genome in a chromosomally integrated form (37) and produces at least the EIA proteins as determined by protein transfer (Western blotting) experiments with extracts from these cells and by antibody reactions with monoclonal sera against E1A protein of Ad2 (Fig. 1). The E1A proteins of Ad5 can also be detected in uninfected and in Ad12-infected BHK297-C131 cells and in Ad2-infected HeLa cells. There is no cross-hybridization with the E1A proteins of Ad12 (data not shown).

Ad12 DNA does not replicate in BHK21 hamster cells (9, 15, 16), and these data have been confirmed (results not shown). Ad12 DNA replication in BHK297-C131 cells is estimated to be 10- to 20-fold reduced compared with replication in permissive human cells (data not shown).

In Ad12-infected BHK21 hamster cells, early but not late Ad12 mRNAs have been detected (21, 24). Upon infection of the Ad5-transformed hamster cell line BHK297-C131 with Ad12, however, late Ad12 RNAs are transcribed (15, 16). In a comparative transcriptional analysis of Ad12-infected human KB and Ad12-infected hamster BHK297-C131 cells, the production of poly(A)⁺ mRNAs has been studied by RNA transfer hybridization (Northern blotting) experiments (2, 19) with DNA segments from the E1 (the EcoRI-C fragment), the L3 (the BamHI-F fragment), the E3 (the PstI-H fragment), the L5 (the PstI-P fragment), and the E4 (the BamHI-E fragment) regions as [32P]-labeled hybridization probes (results not shown). The data presented in Fig. 2 demonstrate that these viral DNA segments are transcribed at 50 h after the productive infection of human KB cells with Ad12 and are also repre-
sent as mRNAs in BHK297-C131 cells at 50 h after Ad12 infection, although in lower quantities (compare nanogram amounts of poly(A)+ RNA applied to the gels). Slight size variations could be due to different lengths of poly(A)+ tails. The differences in the RNA sizes obtained when the EcoRI-C fragment is used as a hybridization probe (Fig. 2) can be explained by the presence of mRNAs from the early E1 and the late pVa, and pIX regions of Ad12 DNA in BHK297-C131 cells. In Ad12-infected KB cells, only the late RNAs from these regions are expressed. It is concluded that in the Ad5-transformed hamster cell line BHK297-C131, which synthesizes at least the E1A proteins of Ad5, Ad12 DNA can replicate and the major early and late transcripts of Ad12 DNA can be detected in these cells as polyadenylated mRNAs. The quantities of the late viral RNAs from the major late transcription unit are up to 20-fold reduced but are clearly detectable in the complementing system. However, transcription of the E3 and the E4 functions is relatively efficient in comparison with transcription in the productive human cell system.

There is no evidence for the synthesis of Ad12 hexon or fiber proteins in the complemented system. In an exemplary study, the presence of late Ad12 proteins, and in particular that of hexon and fiber proteins, was investigated in cytoplasmic and nuclear extracts (Fig. 3) of Ad12-infected KB and Ad12-infected BHK297-C131 cells. Extracts of uninfected KB and uninfected BHK297-C131 cells were used as negative controls. The proteins from these extracts were fractionated by electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes (Western blotting), and reacted with antisera against purified Ad12 virions. In extracts of Ad12-infected BHK297-C131 cells, hexon, fiber, and other late virion structural proteins, which are present in Ad12-infected human KB cells, cannot be detected, even when different methods for protein extraction are applied (data not shown) and when excessive amounts of protein are loaded per polyacrylamide gel slot. The signals apparent in extracts from Ad12-infected BHK297-C131 cells are the same as those in uninfected BHK297-C131 cells (data not shown) and uninfected BHK297-C131 cells and are probably due to unspecific binding (this was not further investigated).

It is concluded that, in the complemented system, late Ad12-specific mRNAs are not translated into detectable amounts of fiber or hexon protein. The possibility that very small amounts of these virion proteins might be present cannot be ruled out. It is conceivable that the lack of the Ad12-specific VA RNA, a viral translational activator, might be responsible for this translational deficiency.

**Authentic Ad12-specific VA RNA is transcribed in Ad12-infected BHK297-C131 cells.** Using the appropriate primer oligodeoxyribonucleotides from the Ad12 VA-RNA gene segment in PCRs subsequent to primer extension with reverse transcriptase, total RNA preparations from Ad12-infected KB and Ad12-infected BHK297-C131 cells were analyzed for the presence of Ad12 VA-RNA sequences. RNAs were pretreated with DNase. Ad12 VA-RNA-specific PCR signals can be elicited with RNA from both Ad12-infected cell types (Fig. 4a), and these signals are identical in size to those obtained with a pAd12 VA-DNA construct containing Ad12 DNA nucleotides between positions 10,053 and 10,900 (13). RNA from uninfected KB and uninfected BHK297-C131 cells did not serve as template for the VA-RNA-specific primers. The Ad12-specific PCR products were directly analyzed for their nucleotide sequences (Fig. 4b), and these sequences were compared with the authentic Ad12 DNA sequence in the VA-RNA segment (32). There is complete sequence identity between the PCR products from Ad12-infected KB and Ad12-infected BHK297-
C131 cells and the VA-RNA gene sequence from Ad12 DNA (Fig. 4b). Thus, the authentic Ad12-specific VA RNA is synthesized in Ad12-infected BHK297-C131 cells, and a lack of this translational activator cannot be the cause for the inability of this complemented system to translate the late Ad12 mRNAs into viral structural proteins.

The Ad12 fiber mRNA synthesized in the complemented system exhibits the authentic nucleotide sequence. The translational block of late Ad12-specific mRNAs in the Ad5-transformed hamster cell line BHK297-C131 could possibly be explained by defects in the nucleotide sequence or splicing mechanisms and in the array of the tripartite leaders of these RNAs. The tripartite leader sequences at the 5' termini of most late adenovirus mRNAs are initiated at the major late promoter (5, 7, 38) and support mRNA translation (1, 6, 14, 20).

We have investigated the Ad12-specific fiber RNAs from Ad12-infected BHK297-C131 cells for such imperfections. Polyadenylated RNAs from these cells were reverse transcribed into cDNAs and cloned via ligated EcoRI and XhoI linker sequences, as described in Materials and Methods. Fiber-specific cDNAs were selected by hybridization to the 32P-labeled PsI-P fragment (see the map in Fig. 2), and the nucleotide sequences of several of these cDNA clones were determined. The same experiments were performed with RNA from Ad12-infected human KB cells as positive references.

The data from several nucleotide sequence determinations are summarized in Fig. 5 and demonstrate that the nucleotide sequence of most reverse transcribed fiber-specific Ad12 mRNAs from Ad12-infected BHK297-C131 cells exhibits the authentic Ad12 nucleotide sequence, which is identical to that in cDNAs derived from RNAs isolated from human KB cells productively infected with Ad12 (Fig. 5). Moreover, in many instances (clones K141, K322, K313, C321, and C361), the mRNAs contain the sequence-perfect tripartite leaders (Fig. 5). In some cases, in RNAs isolated from both Ad12-infected KB and Ad12-infected BHK297-C131 cells, the correct fiber RNA sequence was preceded only by leader sequences 1 and 2 (cDNAs K171 and C281) (Fig. 5a). It is not known what the significance of these possibly aberrant forms of Ad12 fiber RNAs is or whether they can be translated. However, as is evident from Fig. 5a, these aberrant forms have also been seen in productively infected cells. Apparently, there can be a variety of seemingly irregular forms of mRNAs in productively infected cells, as exemplified by the nucleotide sequence of the

FIG. 3. Western blot analysis of late Ad12 proteins in extracts of Ad12-infected human KB cells and of Ad12-infected BHK297-C131 cells. Protein extracts from uninfected KB (KB uninf.), Ad12-infected (68 h postinfection) KB (KB × Ad12), uninfected BHK297-C131 (C131 uninf.) and Ad12-infected BHK297-C131 (C131 × Ad12) cells were prepared according to a published protocol (8). Proteins were fractionated into cytoplasmic (c) and nuclear (n) extracts, and different amounts of proteins were fractionated by electrophoresis on an 8% polyacrylamide gel containing SDS. The proteins were then transferred to nitrocellulose filters and incubated with rabbit anti-Ad12 serum which had been raised against Ad12 virions by conventional methods. As positive controls, proteins of CsCl-purified Ad12 virions lysed in SDS buffer were used (lane Ad12). The structural Ad12 fiber protein is indicated by an arrowhead. The sizes of marker proteins (in kilodaltons) are shown on the right. Proteins in uninfected cells that reacted with the anti-Ad12 serum (internal signals) are shown on the left.

FIG. 4. In Ad12-infected BHK297-C131 cells, the Ad12 VA RNA is expressed. DNase-treated cytoplasmic RNA from uninfected and from Ad12-infected KB (KB and KB × Ad12) and BHK297-C131 (C131 and C131 × Ad12) cells was incubated with primer 1 and Moloney murine leukemia virus reverse transcriptase in a primer extension reaction. Different amounts of the primer extension products (10% [lanes 1] or 20% [lanes 2] of the reaction mixture) were subsequently amplified in a PCR using primers 1 and 2 (see Materials and Methods). (a) The PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by standard ethidium bromide staining. As a marker, the MspI fragments of pBluescript DNA were coelectrophoresed. The sizes (in base pairs) of these fragments are shown on the left. As controls, PCR analyses either without input DNA (no template) or with DNA from a plasmid containing the VA-DNA segment (pAd12 VA) were performed. (b) Nucleotide sequence analyses of the Ad12 VA-RNA-specific PCR products from Ad12-infected KB (VA/KB) or Ad12-infected BHK297-C131 (VA/ C131) cells. The nucleotide sequences were compared with the authentic VA-RNA gene sequence from Ad12 DNA (Ad12) (32).
cDNA clone K162 (Fig. 5a). For technical reasons, most of the cloned fiber-specific cDNAs from either cell system contain only a few nucleotides from leader 1.

The tripartite leader structure of Ad12 fiber-specific mRNAs from Ad12-infected KB and Ad12-infected BHK297-C131 cells has also been assessed by a combination of primer extension and PCR experiments (Fig. 6), as described in Materials and Methods. The data demonstrate that the majority of the PCR products derived from RNAs isolated from both the productive Ad12-infected KB and the complemented Ad12-infected BHK297-C131 systems carry the expected tripartite leader sequences. Leaders other than the major leaders 1, 2, and 3 have not been found in the cDNAs from either population. A minority of mRNA molecules from either cell system is equipped with the truncated leader 1-plus-leader 2 array.

Since atypical leader combinations on Ad12 fiber mRNAs seem at least as abundant in human cells productively infected with Ad12 as in Ad12-infected BHK297-C131 cells, the complementing system, it is unlikely that the structure or the sequence of the Ad12 fiber RNA in BHK297-C131 cells is responsible for the inability of this virus-cell system to produce fiber proteins.

**Conclusions.** We have demonstrated that in the hamster cell line BHK297-C131, a BHK cell line derivative containing in an integrated form the left 18.7% of the Ad5 genome and expressing at least the E1A region of Ad5 DNA, Ad12 DNA replication can proceed. In Ad12-infected BHK297-C131 cells, early (E1, E3, and E4) and late (L3, L5, pIVa, and pIX) regions of the Ad12 genome are transcribed, although in
reduced but clearly detectable amounts in comparison with the same regions transcribed in productively Ad12-infected human KB cells. In BHK21 cells devoid of integrated adenovirus genome fragments, Ad12 DNA replication and late gene transcription cannot be detected by the most sensitive techniques available (9, 15, 16). In spite of the presence of apparently nucleotide sequence-perfect, polyadenylated late Ad12 mRNAs, which are, at least in part, equipped with the authentic tripartite leader sequence, the synthesis of Ad12 fiber (or hexon) protein cannot be demonstrated in the complementing Ad12-infected BHK297-C131 system. The translational activator Ad12 VA RNA is also produced with the authentic sequence in the complementing system. It is, therefore, unlikely that the structure of the mRNA or VA RNA in Ad12-infected BHK297-C131 cells prohibits efficient translation in this system. The apparent translational block of the late Ad12 mRNAs in the complementing system cannot simply be explained by the considerably reduced amounts of at least some of these RNAs, because the E1-specific RNAs derived from the integrated Ad5 DNA are transcribed at comparably low or even lower levels (data not shown) but are translated. Nevertheless, the Ad5 E1-specific mRNAs are efficiently translated, and E1 proteins can be readily detected by Western blotting (Fig. 1). The possibility that minute amounts of virus structural proteins are synthesized cannot be completely ruled out. More likely, cellular functions, either structural or enzymatic, which are required for the efficient translation of late Ad12 RNAs may be absent in hamster cells. The presence of a translational inhibitor is also a possibility.

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