Comparison of Human and Monkey Cells for the Ability To Attenuate Transcripts That Begin at the Adenovirus Major Late Promoter

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Late transcription from the adenovirus major late promoter can terminate prematurely at a site 182 to 188 nucleotides downstream. Experiments have been designed, with run-on transcription in nuclei in vitro or riboprobe protection of RNA obtained both in vivo and in vitro, that demonstrate that the ratio of attenuator RNA to readthrough RNA is greater in monkey cells (CV-1) than in human cells (HeLa). This may explain, in part, why the human adenoviruses replicate more poorly in CV-1 cells than in HeLa cells. A mutant adenovirus that replicates better than wild-type virus in monkey cells produces less of the attenuator RNA than wild-type adenovirus does in monkey cells. Monkey cell extracts have been shown to contain a factor that, when added to human cell extracts transcribing adenovirus DNA in vitro, increases the production of attenuator RNA in these reactions. These observations help to explain a portion of the block to the production of infectious adenoviruses in monkey cells.

The human adenoviruses replicate poorly in most cell lines derived from African green monkey kidney cells (6). An important contribution to this block in the production of infectious virus is the reduction or inhibition of late viral gene transcription, in which some of the late gene families are more severely affected than others (6). This premature termination of transcription appears to occur more frequently during abortive infections of monkey cells than in productively infected cells (3). At 182 to 188 nucleotides (nt) downstream from the major late promoter (MLP) in the adenovirus type 2 (Ad2) genome is an attenuation signal that results in the production of attenuated RNA of that length (10). The experiments reported here were designed to test the hypothesis that attenuation at this site differs in productively and abortively infected cells and this contributes to the reasons why adenoviruses replicate so poorly in these monkey cells.

To examine the levels of attenuator RNAs in monkey (CV-1) or human (HeLa) cells, these cells were infected with Ad2 at a multiplicity of infection (MOI) of 3, 15, or 30 PFU per cell. At 20 h postinfection, nuclei were isolated from these cells and were incubated with a labeled ribonucleotide triphosphate ([α -³²P]UTP) in a buffer that permits RNA synthesis to continue on DNA templates initiated in vivo (see reference 3 for this procedure). Run-on labeled RNA was extracted from these nuclei, and adenovirus-specific RNA was selected by hybridization to a DNA probe that spanned the MLP from 65 nt upstream through 495 nt downstream of this transcription start site. This RNA was eluted from the DNA and was analyzed by acrylamide gel electrophoresis as described previously (10). Figure 1 presents the autoradiographs from these experiments. Figure 1A demonstrates that CV-1 cells infected at an MOI of 15 (CV-1 infected $\times 5$, Fig. 1A) contain the attenuator RNA at the 182- to 185-nt position but uninfected cells and CV-1 cells infected at an MOI of about 3 PFU per cell show no evidence of this RNA. The attenuator RNA was either below the level

of detection or was not produced at low MOIs. CV-1 cells infected at 30 PFU per cell (Fig. 1B, CV-1 [$\times 10$]) and HeLa cells grown in suspension (HeLa-S infected at 30 PFU per cell [$\times 10$] in Fig. 1B) or on the surface of a culture dish (Fig. 1B, HeLa-F) were examined for attenuator RNA by this same procedure. At 30 PFU per cell, the 182- to 185-nt RNA species was readily detected in CV-1 cells but not in HeLa cells (Fig. 1B). At an MOI of 90 PFU per cell, attenuator RNA was readily detected in HeLa cells (Fig. 1C). The detection or appearance of attenuator RNA was dependent upon the MOI, and at the same MOI (30 PFU per cell, Fig. 1B), this RNA was produced in CV-1 cells but not HeLa cells.

A second, independent experimental approach was used to confirm these observations. Nuclei were obtained from Ad2-infected CV-1 or HeLa cells (MOIs of 30 and 90, respectively), and transcription rates were measured by labeling run-on transcripts in vitro (as in reference 3). The labeled RNA was hybridized to adenovirus DNA probes spanning the attenuation signal or the sequences downstream of that signal to monitor the ratio of these RNAs (see Table 1). After RNase treatment, the labeled RNA levels were quantitated by autoradiography and densitometer measurements. These experiments confirmed that attenuation was twofold higher in abortively infected cells (CV-1) than in productively infected cells (HeLa) (results presented in Table 1).

There are at least two ways to relieve the block to adenovirus replication in monkey cells: by the use of a mutant adenovirus, Ad2 *hr400* (4, 5), which contains a mutation in the adenovirus DNA-binding protein gene, and by the expression of the simian virus 40 large T antigen in monkey cells (C-2 cells were used here [2]). Ad2 was used to infect CV-1 and C-2 cells, and Ad2 *hr400* was used to infect CV-1 cells. At 20 h postinfection, nuclei were isolated and run-on transcription was carried out (10). RNA, selected by hybridization, was then analyzed on polyacrylamide gels as before. Figure 2 shows that the attenuator RNA was detectable in CV-1 cells and about two- to threefold less attenuator

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FIG. 1. Size analyses of RNAs produced in isolated nuclei of uninfected and Ad2-infected HeLa and CV-1 cells. (A) CV-1 cells were infected with MOIs of 3 ($\times 1$) and 15 ($\times 5$) PFU per cell. Uninfected cells serve as a control. (B) CV-1 cells, flat HeLa cells grown on plates (F), and HeLa spinner cells (S) were infected with an MOI of 30 PFU per cell ($\times 10$). (C) HeLa F and S cells were infected with an MOI of about 90 PFU per cell ($\times 30$). Nuclei were isolated 20 h postinfection, and transcription was performed as previously described (10). The resulting RNAs were selected by hybridization to and elution from the Ad2 M13 clone 404 DNA (spanning 65 nt upstream through 495 nt downstream of the MLP). They were immobilized on nitrocellulose filters and were analyzed on 7 M urea-6% acrylamide gels. Marker, *Hpa*II-digested pBR322 DNA. The arrowheads point to the position of the attenuator RNA.

RNA was detected with Ad2 *hr400* in CV-1 cells or Ad2 wild type in C-2 cells (equal amounts of counts per minute were loaded on each lane). The levels of attenuator RNA varied from one experiment to another, but a consistent pattern (see Table 1 for quantitation) showed higher levels of attenuator RNAs in Ad2 wild-type-infected CV-1 cells than in Ad2 wild-type-infected C-2 cells or Ad2 *hr400*-infected CV-1 cells.

Because of this variation between experiments, a totally different experimental approach to this question was used to determine how reproducible this observation was. The synthesis of attenuator RNA in vitro in whole-cell extracts (WCE) prepared from monkey or human cells was studied. WCE prepared from HeLa cells, as described by Manley et al. (7), were transcriptionally active, while similar extracts derived from CV-1 cells were not. When CV-1 extracts were mixed 1:1 with HeLa WCE, RNA polymerase II activity was restored, as reported previously by Roeder and colleagues (1). The RNA produced in these in vitro systems from an MLP-containing DNA template (pSmaF DNA [10]) was analyzed by hybridization to labeled riboprobes. These were synthesized in vitro from an SP-6 promoter from a plasmid shown in Fig. 3C. Two different labeled riboprobes were

TABLE 1. Levels of attenuation measured by in vitro transcription assays

Virus and cells	cpm hybridizing to ^a :		Ratio
	Attenuator fragments	Downstream fragments	
Expt A: run-ons			
Ad2-HeLa	105.9 ± 3.6	34.95 ± 2.4	3.0
Ad2-CV-1	123.7 ± 4.4	17.1 ± 2.3	7.2
<i>hr400</i> -CV-1	105.8 ± 1.7	35.0 ± 2.0	3.0
Ad2-C-2	109.8 ± 3.6	31.1 ± 1.9	3.5
Expt B: WCE transcripts			
HeLa	89.0 ± 2.6	52.0 ± 2.1	1.7
CV-1-HeLa	108.7 ± 2.4	35.15 ± 2.0	3.4

hybridization to fragments

^a Labeled run-on RNAs transcribed in vitro in isolated nuclei (expt A) and labeled run-off RNAs transcribed with WCE (expt B) were hybridized to a DNA blot containing the two DNA fragments indicated in the figure: attenuator fragment (Att. frag.) measures RNA from the promoter to the attenuator, while the downstream fragment measures RNA past the attenuator site. The counts per minute hybridized have been corrected and normalized (i) for the U content of the RNAs corresponding to the fragments used (UTP was used for the in vitro labeling), (ii) for the different lengths of the DNA fragments used to hybridize the RNA, and (iii) assuming the attenuator site was used at nt 185 from the 5' end of the RNA.

prepared (A and B) by cutting the plasmid at either the *Sca*I site (probe B, 157 nt) or the *Bam*HI site (probe A, 185 nt). After hybridization to Ad2 RNA that had initiated at the MLP and RNase treatment, probe A could detect attenuator RNA of 154 nt and readthrough RNA of 166 nt, while probe B detected attenuator RNA at 140 nt and readthrough RNA at 152 nt (see Fig. 3C). Thus, these probes can be used to detect the ratio of attenuator to readthrough RNAs made both in vivo and in vitro. This was first done by extracting unlabeled (steady-state) RNA from Ad2-infected HeLa or CV-1 cells. After hybridization to the riboprobe (probe B), RNase treatment, and analysis on polyacrylamide gels, the riboprobe protected by this RNA was detected by autoradiography, and these results are presented in Fig. 3A. Identical attenuator and readthrough RNAs were detected by this analysis of RNAs extracted from infected HeLa and CV-1 cells. The ratio of attenuator to readthrough RNA was higher in CV-1 cells than in HeLa cells (two- to fourfold higher), confirming the differences observed in Fig. 1. When riboprobe A (Fig. 3C) was used, bands of 154 nt (attenuator RNA) and 166 nt (readthrough RNA) were observed at ratios of attenuator to readthrough RNAs similar to those seen in Fig. 3A (results not presented).

FIG. 2. Size analysis of RNAs produced in isolated nuclei of monkey cells. Left lane, Control Ad2-infected CV-1 cells. Center lane, RNA from CV-1 cells infected with the Ad2 mutant *hr400*. Right lane, RNA from Ad2-infected C-2 cells. Infections were 30 PFU per cell. Transcription, RNA selection, and RNA analysis were as described in the legend to Fig. 1. The arrowhead points to the position of the attenuator RNA.

Next, the riboprobe protection analysis was carried out on RNA synthesized in vitro by WCE and exogenously added Ad2 DNA (8). The unlabeled WCE reaction products were analyzed by hybridization to riboprobe B, and this procedure was followed by RNase treatment. Figure 3B shows that HeLa WCE produce (in vitro) a small level of attenuator RNA and a good deal of readthrough RNA. Mixing this WCE 1:1 with CV-1 WCE increases dramatically the level of attenuator RNA synthesized in these WCE reaction (see also Table 1, experiment B). While these in vitro synthetic reactions (Fig. 3B) do not exactly reflect the in vivo steady-state levels (Fig. 3A), it seems likely that the two assays measure different events (i.e., transcription versus transcription, processing, turnover, transport, etc.). When riboprobe A was used to measure the WCE reaction products, the expected sizes of RNAs were obtained in the expected ratios (results not presented). These results suggest that there is a component in CV-1 cell WCE that enhances the attenuation process in vitro, and this is consistent with the idea that attenuation at the 185-nt site could play a role in contributing to the abortive infection of adenoviruses in these cells.

It is unlikely that the attenuator RNA produced in WCE (riboprobe B, 140 nt; Fig. 3B and C) arises from RNA processing or degradation of the longer readthrough RNA. When a kinetic analysis was performed (with time points), the attenuator RNA appeared prior to the readthrough RNA and it did not accumulate at an enhanced rate after the appearance of the readthrough RNA.

These data demonstrate that the production of attenuator RNA and the reduced readthrough at the Ad2 MLP-attenuator site occur more frequently in abortively infected mon-

FIG. 3. Riboprobe mapping analyses. (A) Mapping of the in vivo RNA. Nuclear RNAs isolated from Ad2-infected HeLa and CV-1 cells were hybridized to the *ScaI* riboprobe and RNase treated as previously described (10). The protected bands were analyzed on a 7 M urea-6% acrylamide gel. (B) Mapping of the RNAs produced in HeLa and CV-1-HeLa WCE. For mapping of these transcripts, transcription conditions were as described, but the reaction contained unlabeled UTP. The amounts of RNA (produced in WCE) that were used in this assay correspond to 10% of the transcription reaction. RNAs were hybridized to the *ScaI* probe and were analyzed as in panel A. (C) Schematic representation of the riboprobe protection assay. The upper panel shows the Ad2 sequences downstream of the MLP used for cloning. The second part shows the *PvuII-HindIII* fragment, cloned in a pGEM-1 vector, used for in vitro production of labeled riboprobes. The bottom panel shows the expected sizes of the runoffs and the riboprobes protected by the attenuated RNA and by the full transcript.

key cells than in productively infected human or monkey cells. This was shown by using two different experimental methods, hybridization selection and analysis on run-on RNAs or riboprobe protection and analyses testing RNA produced in vivo and in vitro. The demonstration of attenuator RNA in either monkey or human cells was dependent upon the MOI in these cells. Whether this is just a difference in the sensitivity of detection of attenuator RNA or is a qualitative affect of the MOI remains unclear. The contribution of attenuation, at the attenuator site near the MLP, to the overall inhibition of the production of infectious virus (1,000-fold) may be only 2- to 3-fold, based upon the levels of attenuator RNA in Ad2 wild type versus Ad2 *hr400* or Ad2 wild type in C-2 cells (Fig. 2). An additional two- to threefold increase in premature termination of late transcription, along the 7-kilobase site of the Ad2 genome downstream from the attenuation site, in abortively infected cells has been dem-

onstrated by Klessig and his colleagues (3). While this adds up to a modest (four- to ninefold) contribution to the inhibition of virus production, it remains clear that different methods of analysis have provided different quantitative results (see Fig. 1 to 3).

The use of WCE and in vitro transcription indicates that monkey WCE can contribute a factor that enhances the production of attenuator RNA in vitro and alters the ratio of attenuator to readthrough RNA (Fig. 3). This factor may interact with the DNA-binding protein which has recently been shown to bind to attenuator RNA (9). This could explain the role of DNA-binding protein *hr400* in relieving the inhibition of productive infection in monkey cells. If this explanation is correct, then the use of the attenuator site 182 to 188 base pairs downstream from the MLP, where both the DNA-binding protein and the termination factor act, would have to be a major reason (not a two- to threefold effect) why adenoviruses fail to produce infectious virus in monkey cells. Clearly, more information is required before this problem is solved.

This work was supported by Public Health Service grant CA14995 from the National Institutes of Health; by a grant from The United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel; by The Rockefeller-WIF Collaboration Trust Fund; and by the Minerva Foundation, Munich, Federal Republic of Germany.

LITERATURE CITED

1. Abmayr, S. M., D. L. Feldman, and R. Roeder. 1985. In vitro stimulation of specific RNA polymerase II-mediated transcription by the pseudorabies virus immediate early protein. *Cell* 43:821-829.
2. Grodzicker, T., C. Anderson, P. A. Sharp, and J. Sambrook. 1974. Conditional lethal mutants of adenovirus 2-simian virus 40 hybrids. I. Host range mutants of Ad2⁺ND1. *J. Virol.* 13: 1237-1244.
3. Johnston, J. M., K. P. Anderson, and D. F. Klessig. 1985. Partial block to transcription of human adenovirus type 2 late genes in abortively infected monkey cells. *J. Virol.* 56:378-385.
4. Klessig, D. F. 1977. Isolation of a variant of human adenovirus serotype 2 that multiplies efficiently on monkey cells. *J. Virol.* 21:1243-1246.
5. Klessig, D. F. 1984. Adenovirus-simian virus 40 interactions, human adenovirus growth in monkey cells, p. 426-435. *In* H. S. Ginsberg (ed.), *The adenoviruses*. Plenum Publishing Corp., New York.
6. Klessig, D. F., and J. A. Hassell. 1978. Characterization of a variant of human adenovirus type 2 which multiplies efficiently in simian cells. *J. Virol.* 28:945-956.
7. Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gefter. 1980. DNA dependent transcription of adenovirus genes in a soluble whole cell extract. *Proc. Natl. Acad. Sci. USA* 77: 3855-3859.
8. Melton, D. A., P. A. Kreig, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035-7057.
9. Seiberg, M., Y. Aloni, and A. J. Levine. 1989. The adenovirus type 2 DNA-binding protein interacts with the major late promoter attenuated RNA. *J. Virol.* 63:1134-1141.
10. Seiberg, M., M. Kessler, A. J. Levine, and Y. Aloni. 1987. Human RNA polymerase II can prematurely terminate transcription of the adenovirus type 2 late transcription unit at a precise site that resembles a prokaryotic termination signal. *Virus Genes* 1:97-116.