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Control of Adenovirus Early Gene Expression: Posttranscriptional Control Mediated by Both Viral and Cellular Gene Products

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An adenovirus type 5 host range mutant (hr-1) located in region E1A and phenotypically defective in expressing viral messenger ribonucleic acid (RNA) from other early regions (Berk et al., Cell 17:935-944, 1979) was analyzed for accumulation of viral RNA in the presence of protein synthesis inhibitors. Nuclear RNA was transcribed from all early regions at the same rate, regardless of whether the drug was present or absent. As expected, low or undetectable levels of RNA were found in the cytoplasm of hr-1-infected cells compared with the wild-type adenovirus type 5 in the absence of drug. When anisomycin was added 30 min before hr-1 infection, cytoplasmic RNA was abundant from early regions E3 and E4 when assayed by filter hybridization. In accordance, early regions E3 and E4 viral messenger RNA species were detected by the S1 endonuclease mapping technique only in hr-1-infected cells that were treated with the drug. Similar results were obtained by in vitro translation studies. Together, these results suggest that this adenovirus type 5 mutant lacks a viral gene product necessary for accumulation of viral messenger RNA, but not for transcription. It is proposed that a cellular gene product serves as a negative regulator of viral messenger RNA accumulation at the posttranscriptional level.

At early times after adenovirus infection, at least five regions of the viral genome are transcribed into cytoplasmic ribonucleic acid (RNA) (2, 18). Studies with host range mutants have suggested that a major element of genetic control exists in early region E1A (1.5 to 4.5 map units) of the viral deoxyribonucleic acid (DNA). Jones and Shenk (11) isolated an adenovirus type 5 (Ad5) mutant, dl312, with a deletion from 1.5 to 4.5 map units, which was defective both for growth in HeLa cells and for transformation of rat cells. Furthermore, dl312 was defective in the accumulation of cytoplasmic early messenger RNA (mRNA) (12). However, since nuclear RNA was detected at a low level in dl312-infected HeLa cells, the defect in the mutant may be at a posttranscriptional level such as processing, transport, or stability of the RNA.

Another Ad5 mutant, hr-1, also unable to replicate in HeLa cells and defective in transformation, was isolated by Harrison et al. (10). Recently, the lesion in the hr-1 mutant was mapped between 1.3 and 3.7 units of the Ad5 genome by marker rescue (8). Berk et al. (1) examined the accumulation of viral RNA in hr-1-infected HeLa cells by S1 endonuclease mapping. Both cytoplasmic and nuclear RNA from early regions E1B, E2, E3, and E4 were undetectable in cells infected with the mutant. However, early region E1A RNAs were present in the nucleus and the cytoplasm. These investigators concluded that an early region E1A gene product, which is defective in the hr-1 mutant, is required for either the initiation of transcription or stabilization of early viral nuclear RNA.

The regulation of viral mRNA expression has been studied by using inhibitors of protein synthesis (13, 16). It was found that viral mRNA accumulation was depressed when inhibitors were added shortly before Ad2 infection of HeLa cells. This observation, along with other evidence, led Persson et al. (16) to propose a model in which a protein coded by early region E1 regulates the accumulation of viral mRNA by inactivating a cellular component normally responsible for turnover of viral mRNA. In a separate report, we presented evidence that the protein(s) encoded in early region E1 primarily controls the early region E4 of the viral genome (H. Persson, M. G. Katze, and L. Philipson, J. Virol., in press).

The present study examined the control of viral mRNA accumulation in hr-1-infected HeLa cells by using inhibitors of protein synthesis. The results are consistent with the hypothesis outlined by Persson et al. (16) and strongly

suggest that the hr-1 mutant lacks a mechanism to counteract the negative cellular control.

MATERIALS AND METHODS

Cells and virus. HeLa cells were grown in suspension at concentrations of 3×10^5 to 5×10^5 cells per ml in Eagle spinner medium containing 7% calf serum. Wild-type Ad5 was grown and purified from infected HeLa cells as previously described (18). Ad5 mutant hr-1 (10) was propagated in the permissive Ad5-transformed 293 cells (9). The hr-1 mutant was titrated on both 293 and HeLa cells. The mutant virus had titers on HeLa cells 10^3 to 10^4 times lower than the titers on 293 cells.

Infection and labeling conditions. HeLa cells were infected with wild-type Ad5 and mutants at a multiplicity of 1 plaque-forming unit per cell. Cytoplasmic RNA was prepared from HeLa cells labeled with [^{3}H]uridine (50 μCi/ml) 2 to 5 h postinfection by the method of Brawerman et al. (4). Nuclear RNA was prepared from cells pulse-labeled with [^{3}H]uridine (500 μCi/ml) for 5 min at 5 h postinfection by the procedure of Smith et al. (20).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and in vitro translation. The procedures for translation in the rabbit reticulocyte cell-free system have been described previously (17). Virus-specific RNA was selected on Ad5 DNA or purified restriction enzyme fragments (18) bound to nitrocellulose filters as described by McGrogan et al. (14). Eluted RNA was then translated in the mRNA-dependent reticulocyte system. Samples were analyzed on 13% sodium dodecyl sulfate-polyacrylamide slab gels as described previously (15, 17). The gels were analyzed by fluorography (3).

S1 endonuclease mapping of mRNA. The method originally described by Berk and Sharp (2) and modified by Persson et al. (16) was followed for S1 endonuclease mapping of mRNA. Briefly, 20 μg of cytoplasmic RNA from each sample was hybridized in a high concentration of formamide (5) to 1 μg of Ad5 DNA. The samples were treated with S1 endonuclease (225 U) and separated on a 2.0% alkaline agarose gel. The gels were blotted onto nitrocellulose sheets (21) and hybridized to ^{32}P-labeled nick-translated probes (19).

Filter hybridization. Equal amounts of nuclear or cytoplasmic RNA were hybridized for 16 h at 65°C to nitrocellulose filters containing *Sal*I and *Eco*RI fragments of Ad5 derived from 10 μg of viral DNA. After hybridization the filters were washed extensively and treated with ribonuclease A (20 μg/ml). The amount of labeled RNA hybridized was quantitated by liquid scintillation counting.

RESULTS

Accumulation of cytoplasmic viral RNA in cells infected with Ad5 host range mutant hr-1. Host range mutants with either defined deletions or point mutations in early region E1A have been constructed (10, 11). At low multiplicities of infection these mutants do not accumulate cytoplasmic RNA from viral early region E2, E3, or E4 (1, 12) in HeLa cells permissive for the wild-type virus. We examined the accumulation of early viral RNA in HeLa cells infected with a host range mutant in the presence of inhibitors of protein synthesis. Previous studies with inhibitors added before or after infection of human cells with wild-type Ad2 have shown that inhibitors effect the accumulation of early viral mRNA (13, 16).

In the present experiments HeLa cells were infected with the host range mutant hr-1 with and without the protein synthesis inhibitor anisomycin added at 30 min before infection. To avoid late expression from the control wild-type virus all samples were collected at 5 h after infection at a time when the E2 region is not yet fully expressed. We have therefore not included the E2 region in our analysis. The cells were labeled with [^{3}H]uridine from 2 to 5 h postinfection, and cytoplasmic RNA was isolated. The labeled RNA was subsequently hybridized to nitrocellulose filters containing different fragments of Ad5 DNA. As expected, the host range mutant hr-1 accumulated substantially less early viral RNA from region 3 and 4 than did the wild-type virus (Table 1). The addition of anisomycin 30 min before infection, however, allowed the accumulation of early viral RNA from infected cells. The amount of mutant early viral RNA accumulated under these conditions reached a level comparable to that in the wild-type infections.

Rate of transcription in mutant-infected cells. Infected cells were pulse-labeled with [^{3}H]uridine for 5 min at 5 h after infection. The nuclear RNA was extracted and hybridized to filter-bound Ad5 DNA fragments representing different early viral transcription units. The results for wild-type Ad5 and the hr-1 mutant

TABLE 1. *Effect of anisomycin on the accumulation of cytoplasmic early viral mRNA in mutant-infected cells*

Prepn	RNA hybridized (cpm)[a]	
	E3	E4
Wild-type Ad5	6,047	6,598
hr-1	802	1,648
hr-1 with anisomycin[b]	7,152	9,090

[a] Equivalent amounts of early RNA were hybridized to filters as described in the text. The probe for early region E4 was the *Eco*RI-C fragment of Ad5 (84.0 to 100.0 map units), and the probe for early region E3 was the *Eco*RI-B fragment (75.9 to 84.0 map units). A background of approximately 50 cpm to filters without DNA has been subtracted from each value.

[b] Ad5 mutant-infected cells were maintained in the presence of 100 μM anisomycin (Pfizer Laboratories) from 30 min before infection until 5 h postinfection.

TABLE 2. *Rate of transcription in Ad5- and hr-1-infected HeLa cells*

Prepn	RNA hybridized (cpm)[a]		
	E1	E3	E4
Ad5	793	575	142
hr-1	1,219	598	381
hr-1 with anisomycin[b]	770	100	280

[a] Ad5- and hr-1-infected cells were pulse-labeled for 5 min at 10^7 cells per ml with 500 μCi of [^{3}H]uridine per ml at 5 h postinfection. Equivalent amounts of nuclear RNA were hybridized to filters as described in the text. The probes of Ad5 DNA used were: early region E1, *Sal*IB (0 to 26.0 map units); early region E3, *Eco*RI-C (75.9 to 84.0 map units); early region E4, *Eco*RI-B (84.0 to 100.0 map units). A background of approximately 40 cpm to filters without DNA has been substracted from each value.

[b] hr-1 mutant-infected cells were maintained in the presence of 100 μM anisomycin from 30 min before infection until 5 h postinfection.

virus are shown in Table 2. Nuclear RNA from transcription units E1A, E1B, E3, and E4 was detected in hr-1-infected cells. Furthermore, the amount of labeled nuclear RNA was comparable to the amount obtained with the wild-type virus. The rate of transcription of hr-1 nuclear RNA from early regions E1A, E1B, E3, and E4 was not increased when anisomycin was present throughout the time of labeling (Table 2). The results with the hr-1 mutant suggest that the increased accumulation of viral cytoplasmic RNA in the presence of anisomycin is not caused by an increased rate of transcription in the presence of the drug.

S1 blot analysis of cytoplasmic RNA from mutant-infected cells. Cytoplasmic RNA was isolated at 5 h postinfection from cells treated with anisomycin 30 min before or 2 h after infection with the hr-1 mutant. RNA was also isolated from cells not treated with anisomycin and from wild type-infected cells. The cytoplasmic RNA was hybridized to Ad5 DNA, treated with S1 endonuclease, separated on an alkaline agarose gel, and blotted onto nitrocellulose sheets. The filters were subsequently hybridized to ^{32}P-labeled DNA fragments of Ad5 DNA representing different regions. The results for early region E3 are shown in Fig. 1A. No viral RNA from region E3 was detected in hr-1-infected cells in the absence of anisomycin. However, when anisomycin was present, substantial amounts of early region E3 RNA were detected. Four major RNA species were detected, corresponding in size to the ones previously identified from this region after wild-type infection (1). The same four RNA species were detected in wild type-infected cells. S1 endonuclease analysis of region E4 RNA also showed that the drug treatment allowed the accumulation of viral RNA from hr-1-infected cells. As with region E3, no hr-1 RNA was detected in the absence of the drug (Fig. 1B). Furthermore the major RNAs from region E4 accumulated in drug-treated, hr-1-infected cells had the same sizes as the RNA species obtained from wild type-infected cells.

In vitro translation with RNA prepared from hr-1-infected cells. To test whether the RNA that accumulated in hr-1-infected cells in the presence of anisomycin was functional, the

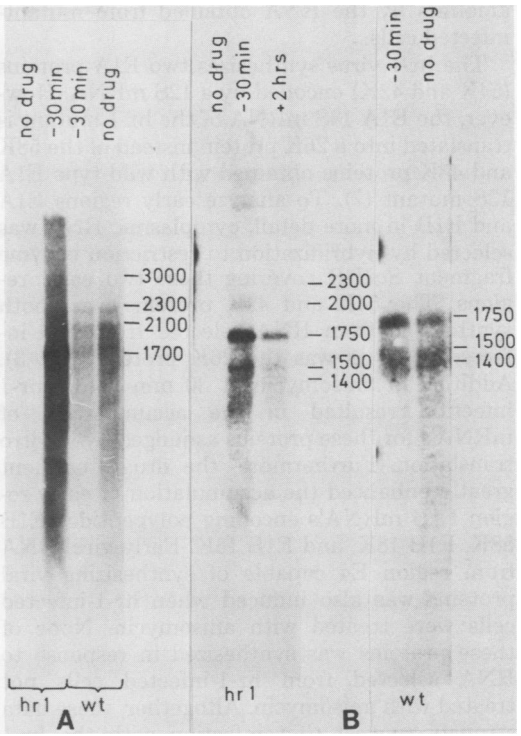

FIG. 1. *S1 endonuclease analysis of cytoplasmic RNA prepared from cells infected with host range mutants. Cytoplasmic RNA was prepared from wild-type Ad5- or hr-1-infected HeLa cells at 5 h postinfection. The cells were maintained in the absence of anisomycin (no drug) or in the presence of anisomycin (100 μM) from 30 min before infection (−30 min) or from 2 h postinfection (+2 hrs). The RNA was hybridized in a high concentration of formamide to Ad5 DNA, treated with S1 endonuclease, and separated on a 2.0% alkaline agarose gel. The gel was blotted to a nitrocellulose sheet and hybridized to restriction enzyme fragments of Ad5 DNA labeled with ^{32}P by nick translation. The filter was then washed and analyzed by autoradiography. (A) Early region E3 Ad5 DNA probe: EcoRI-C (75.9 to 84.0 map units). (B) Early region E4 Ad5 DNA probe: EcoRI-B (84.0-100 map units). The virus used is indicated below the gels. wt, Wild type.*

RNA was selected by hybridization to Ad5 DNA. The selected RNA was then translated in a cell-free, protein-synthesizing system. RNA prepared from hr-1-infected cells showed little or no synthesis of early viral proteins. In contrast, RNA prepared from hr-1-infected cells maintained in the presence of anisomycin directed the synthesis in vitro of a complex pattern of early viral proteins (Fig. 2). These proteins were also detected when RNA from wild type-infected cells was used to program the cell-free system. However, several proteins, such as E1B 65K and E3 16K, were synthesized in greater amounts by the RNA obtained from mutant-infected cells.

The hr-1 virus synthesizes two E1A proteins (54K and 42K) encoded by a 12S mRNA. However, the E1A 13S mRNA of the hr-1 mutant is translated into a 26K protein instead of the 58K and 48K proteins obtained with wild-type E1A 13S mutant (7). To analyze early regions E1A and E1B in more detail, cytoplasmic RNA was selected by hybridization to restriction enzyme fragment *Sal*I-B covering these two early regions. The 54K and 42K proteins were both synthesized from RNA selected from hr-1-infected cells, as was the 26K protein (Fig. 3). Addition of anisomycin at 30 min before hr-1 infection resulted in the accumulation of mRNA's for these proteins as judged by in vitro translation. Furthermore, the drug treatment greatly enhanced the accumulation of early region E1B mRNA's encoding polypeptides E1B 58K, E1B 18K, and E1B 15K. Early viral RNA from region E4 capable of synthesizing viral proteins was also induced when hr-1-infected cells were treated with anisomycin. None of these proteins was synthesized in response to RNA selected from hr-1-infected cells not treated with anisomycin. Altogether, these data strongly suggest that infection with the hr-1 mutant in the presence of anisomycin allows the accumulation of functional viral mRNA's from at least three early regions.

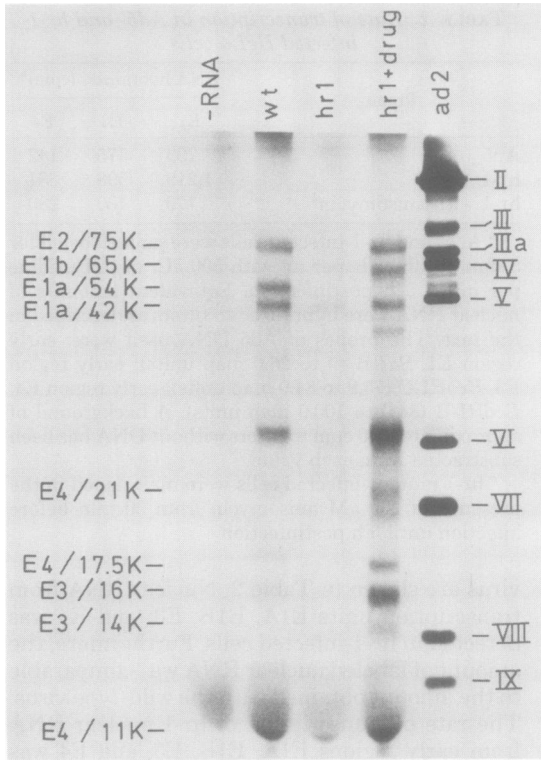

FIG. 2. *In vitro translation with hybridization-selected RNA prepared from hr-1-infected cells. Cytoplasmic RNA was prepared at 5 h postinfection from HeLa cells infected with wild-type Ad5 or hr-1 virus. Cells infected with hr-1 were also treated with anisomycin (100 μM) from 30 min before infection throughout the experiment (hr-1 + drug). The RNA was hybridized to filters containing Ad5 DNA, and the hybridized RNA was translated in the mRNA-dependent reticulocyte cell-free system. The translational products were analyzed on a 13% sodium dodecyl sulfate-polyacrylamide gel. The gel was analyzed by fluorography. Abbreviations: −RNA, no RNA added to the cell-free system; ad2, [^{35}S]methionine-labeled Ad2 marker virus.*

DISCUSSION

Host range mutants in early region E1A of the Ad5 genome have been isolated previously (10, 11). These mutants do not accumulate early viral mRNA from regions E2, E3, and E4, suggesting that viral gene products from early region E1A control the expression of RNA from other early viral regions (1, 12). The deletion mutants (such as dl312) lack the entire early region E1A and therefore do not express any gene products from this region. On the other hand, the host range mutants belonging to complementation group I express some gene products from early region E1A. The hr-1 mutant, which belongs to complementation group I, expresses a functional E1A 12S mRNA encoding a 54K and a 42K protein. However, the E1A 13S mRNA is not translated into a 58K and a 48K protein as in the wild-type virus, but is instead translated into a 26K protein (7). This suggests that the hr-1 mutant contains a mutation introducing a stop codon for protein synthesis in the E1A 13S mRNA. Additional lesions which have not yet been detected in this mutant may exist. Berk et al. (1) suggested that the defect in the hr-1 mutant is at the level of transcription since the mutant also fails to accumulate nuclear RNA

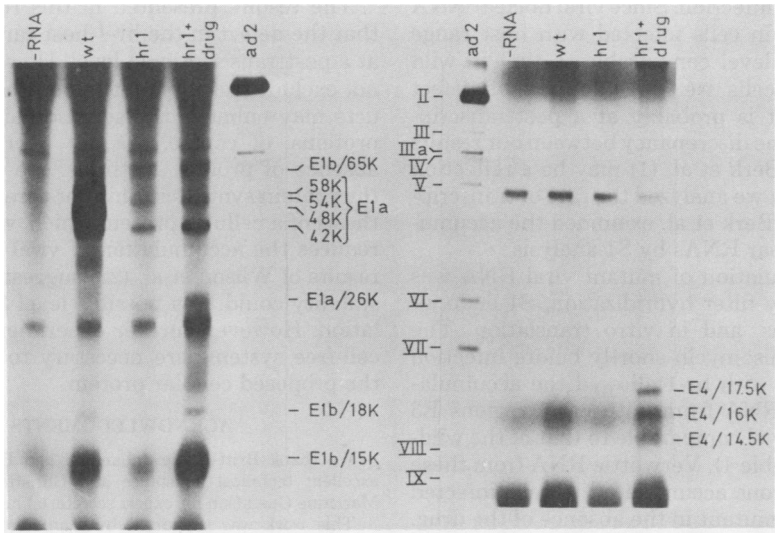

FIG. 3. *In vitro translation with RNA selected by hybridization to different early regions of the Ad5 genome. Cytoplasmic RNA was prepared from HeLa cells infected with wild-type Ad5 or hr-1 virus. The hr-1-infected cells were also treated with anisomycin (100 μM) from 30 min before infection to the time of harvest at 5 h postinfection (hr-1 + drug). The RNA was hybridized to filters containing fragment SalI-B of Ad5 DNA (0 to 26.0 map units, left panel) or to filters containing fragment EcoRI-B of Ad5 DNA (84.0 to 100 map units, right panel). The hybridized RNA was translated in the reticulocyte cell-free system, and the translational products were analyzed on a 13% sodium dodecyl sulfate-polyacrylamide gel. The gel was analyzed by fluorography. The SalI-B DNA probe represents early regions E1A and E1B. The EcoRI-B probe represents early region E4. Abbreviations: −RNA, no RNA added to the cell-free system; ad2, [^{35}S]methionine-labeled Ad2 marker virus.*

from other early regions. On the other hand, small amounts of nuclear RNA were found to accumulate in dl312-infected cells (12). The present results suggest that the defect is at a posttranscriptional level.

When added after viral infection, inhibitors of protein synthesis enhance the accumulation of early viral mRNA (6). Furthermore, this enhancement may be caused by an increased mRNA stability since the half-life of early viral mRNA is prolonged in the presence of inhibitors of protein synthesis (22). Experiments in which anisomycin was added before Ad2 infection of HeLa cells showed a reduced accumulation of functional mRNA from early regions E2, E3, and E4 (13, 16). These results were interpreted to mean that a viral protein from early region E1A controls the accumulation of functional viral mRNA's from other early regions. In accordance, the rate of transcription from the early transcription units was only weakly affected when anisomycin was added before infection, whereas mRNA accumulation was strongly depressed (16). On the basis of these results we suggested that the E1A proteins are involved at a posttranscriptional level, and the results of Wilson et al. (22) led us to propose that the E1A proteins control mRNA accumulation at the level of RNA stability. A model was introduced in which a viral protein encoded in early region E1A inactivates a cellular protein that degrades viral mRNA. The E1A viral protein involved in the stabilization of viral mRNA would then be absent or not functional in the host range mutants, thereby allowing the cellular product to degrade viral mRNA. The addition of anisomycin before infection of HeLa cells with the mutants would prevent the synthesis of the cellular destabilizing protein. In this way, the accumulation of mutant viral mRNA should be facilitated. The present study was designed to test this hypothesis.

If our hypothesis is correct, the defect in the host range mutants should act at a posttranscriptional level. We first determined the rate of transcription by labeling infected cells for 5 min with [^{3}H]uridine and measuring the amount of label incorporated into viral nuclear RNA by filter hybridization to viral DNA fragments representing different early viral regions. The results (Table 2) show that nuclear RNA is transcribed in HeLa cells infected with the hr-1 mutant from early regions E1, E3, and E4 at the same rate as in cells infected with the wild-type virus. The rate of transcription was not increased in hr-1-infected cells treated with anisomycin

shortly before infection. Since viral nuclear RNA was detected in cells infected with host range mutants at a level comparable to that in wild type-infected cells, we conclude that the defect in the mutant is probably at a posttranscriptional level. The discrepancy between our results and those of Berk et al. (1) may be a reflection of the fact that we analyzed the rate of transcription, whereas Berk et al. examined the accumulation of nuclear RNAs by S1 analysis.

The accumulation of mutant viral RNA was determined by filter hybridization, S1 endonuclease analysis, and in vitro translation. The addition of anisomycin shortly before infection of HeLa cells with hr-1 allowed the accumulation of viral RNA from both early regions E3 and E4 at a level comparable to that of the wild-type virus (Table 1). Very little RNA from these two early regions accumulated in cells infected with the hr-1 mutant in the absence of the drug.

The viral RNA accumulated in hr-1-infected cells maintained in the presence of anisomycin had the same structure as RNA obtained from wild type-infected cells, as revealed by S1 mapping analysis (Fig. 1). Early viral RNA accumulated from regions E3 and E4 in hr-1-infected cells treated with anisomycin 30 min before or 2 h after infection (Fig. 1). The RNA from cells treated from 30 min before infection appeared to be more abundant than RNA prepared from cells treated with anisomycin from 2 h after infection. This result may reflect a level of the cellular destabilizing protein in cells treated with anisomycin from 30 min before infection that is lower than the level in cells treated from 2 h postinfection.

The RNA accumulated in hr-1-infected cells maintained in the presence of anisomycin was functional when assayed by in vitro translation (Fig. 2). Hybridization selection of RNA prepared from hr-1-infected cells maintained in the absence or presence of anisomycin to DNA fragment *Sal*I-B, representing early regions E1A and E1B, followed by in vitro translation proved the mutant character of the hr-1 virus. The E1A 58K and 48K proteins were not synthesized by either of these two hr-1 RNA preparations, but instead we detected a 26K protein. This result is in agreement with the results of Esche et al. (16), and it demonstrated that the virus used in this study had the expected lesion in the E1A 13S mRNA.

However, viral proteins from early regions E1B, E2, E3, and E4 were readily detected from hr-1 RNA preparations maintained in the presence of anisomycin. The amount of hr-1 viral mRNA accumulated under these conditions reached a level comparable to that of the wild-type virus.

The results presented in this report suggest that the defect in the hr-1 host range mutant is at a posttranscriptional level. However, we cannot exclude the possibility that other E1A products may enhance transcription since the E1A proteins, of course, are not expressed in the absence of protein synthesis. We propose that the protein synthesis inhibitors prevent the synthesis of a cellular protein which, when present, reduces the accumulation of viral mRNA. The results of Wilson et al. (22) suggest that mRNA stability could be a possible level for this regulation. However, further experiments involving cell-free systems are necessary to characterize the proposed cellular protein.

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