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The Adenovirus L4 100-Kilodalton Protein Is Necessary for Efficient Translation of Viral Late mRNA Species

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When screening a number of adenovirus type 5 (Ad5) temperature-sensitive mutants for defects in viral gene expression, we observed that H5ts1-infected 293 cells accumulated reduced levels of newly synthesized viral late proteins. Pulse-labeling and pulse-chase experiments were used to establish that the late proteins synthesized in H5ts1-infected cells under nonpermissive conditions were as stable as those made in Ad5-infected cells. H5ts1-infected cells contained normal levels of viral late mRNAs. Because these observations implied that translation of viral mRNA species was defective in mutant virus-infected cells, the association of viral late mRNAs with polyribosomes was examined during the late phase of infection at a nonpermissive temperature. In Ad5-infected cells, the majority of the viral L2, L3, L4, pIX, and IVa_2 late mRNA species were polyribosome bound. By contrast, these same mRNA species were recovered from H5ts1-infected cells in fractions nearer the top of polyribosome gradients, suggesting that initiation of translation was impaired. During the late phase of infection, neither the polyribosome association nor the translation of most viral early mRNA species was affected by the H5ts1 mutation. This lesion, mapped by marker rescue to the L4 100-kilodalton (kDa) nonstructural protein, has been identified as a single base pair substitution that replaces Ser-466 of the Ad5 100-kDa protein with Pro. A set of temperature-independent revertants of H5ts1 was isolated and characterized. Either true reversion of the H5ts1 mutation or second-site mutation of Pro-466 of the H5ts1 100-kDa protein to Thre, Leu, or His restored both temperature-independent growth and the efficient synthesis of viral late proteins. We therefore conclude that the Ad5 L4 100-kDa protein is necessary for efficient initiation of translation of viral late mRNA species during the late phase of infection.

A characteristic of the late phase of infection of permissive cells by subgroup C adenoviruses is the gradual, but eventually severe, inhibition of cellular protein synthesis (for example, see references 4, 6, 65). Although the synthesis of host cell proteins is grossly impaired from about 18 h after infection under typical conditions, neither the stability nor the in vitro translatability of cellular mRNA species is decreased (for example, see references 5, 30, 45, 68). These observations indicate that adenovirus infection induces the selective translation of viral mRNA species during the late phase. The mechanisms that lead to efficient translation of viral mRNA, but inhibition of cellular protein synthesis, in adenovirus-infected cells have not been fully elucidated. Indeed, it seems likely that a complex interplay of regulatory circuits may be involved: two virus-specific products that influence the efficiency with which viral or cellular mRNA species are translated have been identified, and the synthesis of large quantities of viral late proteins occurs despite a host cell defense mechanism that has the potential to shut down all protein synthesis.

Continued initiation of translation during the late phase of infection requires the production of the small viral RNA, $VA\text{-}RNA_I$ (51, 53, 60). This RNA species limits the activation of a protein kinase, known as DA1 or P1/eIF-2α kinase, which phosphorylates the α-subunit of the translation initiation factor eIF-2 (31, 48, 54, 57). When the α-subunit of eIF-2 is phosphorylated, the limited quantity of eIF-2B, which catalyzes the conversion of GDP bound to eIF-2 to GTP and thus recycling of active eIF-2, is sequestered in a complex with eIF-2 and initiation of translation is inhibited (see reference 28). Thus, a primary role of $VA\text{-}RNA_I$ is to overcome the inhibitory consequences of the activation of the P1/eIF-2α kinase induced by adenovirus infection (see reference 52). However, it has been suggested that $VA\text{-}RNA_I$ might also contribute to the translational discrimination between viral and cellular mRNA species; O'Malley and colleagues (41) made the surprising observation that the extent of phosphorylation of eIF-2α induced by the late phase of infection in the presence of $VA\text{-}RNA_I$ is as high as that known to inhibit protein synthesis completely under other circumstances. To account for this seeming paradox, these authors proposed that the protein synthesis machinery of adenovirus-infected cells is divided between two noncommunicating compartments, one containing $VA\text{-}RNA_I$ and viral mRNA and the second containing cellular (and perhaps viral early) mRNA species but no $VA\text{-}RNA_I$. Translation of the former mRNA species would, therefore, be protected by $VA\text{-}RNA_I$ from the inhibitory consequences of P1/eIF-2α kinase-mediated phosphorylation of the eIF-2α subunit. This mechanism provides an elegant explanation of the selective translation of viral mRNA species and is consistent with the observation that two cell lines in which P1/eIF-2α kinase is not efficiently activated fail to exhibit inhibition of cellular protein synthesis following adenovirus type 2 (Ad2) infection (41).

A second virus-encoded element that governs translational efficiency is the tripartite leader. This sequence of some 200 nucleotides, which is formed by the splicing of three small exons, comprises a 5′ untranslated segment common to the majority of mRNA species synthesized during the late phase of infection, those transcribed from the major late transcription unit (7, 17). The tripartite leader significantly enhances the efficiency with which heterologous mRNA species are translated during the late (8, 10, 12,

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37), but not the early (37), phase of infection. Sequences from all three exons of the leader are required for maximal effect (11, 37), and the leader does not increase translational efficiency when placed at the 3′ end of mRNA (37), suggesting a role in initiation. It has been postulated that the tripartite leader might reduce or eliminate the requirement for the cap-binding eIF-4F complex (20) or facilitate the compartmentalization of viral mRNA with VA-RNA$_I$ (38, 41). The mechanism, or mechanisms, whereby this sequence increases translational efficiency have not, however, been fully elucidated.

Here we report that the translation of viral mRNA species during the late phase of adenovirus infection is regulated by yet a third virus-encoded product: during the late phase of infection, the efficient translation of late, but not typical early, viral mRNA species requires the synthesis of a functional L4 100-kDa nonstructural protein.

MATERIALS AND METHODS

Cells and virus. Ad5 was grown in either HeLa suspension cells or monolayers of A549 cells (25) at 37°C. The Ad5 temperature-sensitive mutant H5ts1 (67) was grown either on A549 cells or on cells of the Ad5-transformed human embryonic kidney 293 line (27) at 33°C. Wild-type revertants of H5ts1 were isolated as individual plaques and grown upon A549 cells at 39°C. Mutant *dl*331, which was obtained from T. Shenk, was grown upon 293 cells at 37°C. Cells of a KB line (32) used in some experiments were obtained from T. Shenk. All cells were maintained in Dulbecco modified Eagle medium supplemented with either 7% calf serum (A549) or 5% fetal calf serum plus 5% calf serum (293; HeLa and KB). Infectivities of wild-type, mutant, and revertant stocks were measured by plaque assay on either HeLa or A549 cells (66). In all experiments, 293, HeLa, or KB cell monolayers at subconfluent densities were infected at an input multiplicity of 20 PFU per cell, based on plaque assay on HeLa cells, and the end of a 1-h absorption period at 37°C was taken at time zero.

DNA sequencing. DNA sequencing was performed by using Sequenase (United States Biochemical Corp.). To locate the H5ts1 mutation precisely, viral DNA isolated from H5ts1-infected A549 cells was cleaved with *Asp*718 and the *Asp*718 E fragment, which encompassed the marker rescue limits of the mutation, and was isolated by separation in and excision from a 1% low-melting-temperature agarose gel. The purified DNA fragment was subsequently recut with *Aat*II and cloned into the plasmid vector pUC18 to generate the plasmid pGT D3. The same procedure was used to generate pGT A3 from wild-type Ad5 DNA. The DNA sequence of H5ts1 was determined by double-stranded sequencing of minipreparations of pGT D3 (72), using a series of oligonucleotide primers that spanned the marker rescue limits of the mutation. The corresponding wild-type plasmid was sequenced for comparison. The DNA sequences of revertants r(*ts*1)1 and r(*ts*1)2 were determined by double-stranded plasmid sequencing of the cloned *Sma*I E fragments from each virus. Sequencing of the five remaining revertants was performed without cloning DNA fragments into plasmid vectors. Viral DNA was isolated by disruption of purified virions with proteinase K and sodium dodecyl sulfate (SDS) followed by phenol and chloroform extractions and ethanol precipitation. A 10-μg portion of purified viral DNA was sequenced in a similar way to the plasmid templates, except that the DNA was heated to 83°C during the denaturation. Primer was added at a 10-fold molar excess. Best results were obtained when [^{32}P]dATP was used in the sequencing reactions.

Protein labeling. Unless otherwise indicated, proteins were labeled for 30 or 50 min at 39 or 33°C, respectively, with 100 μCi of [^{35}S]methionine per ml of Dulbecco modified Eagle medium minus methionine per 60-mm dish. At the end of the labeling period, cells were harvested and suspended in 0.125 M Tris hydrochloride, pH7.4, containing 1.0% (wt/vol) SDS and 5.0% (vol/vol) β-mercaptoethanol (GSB), sonicated until the lysates were no longer viscous, and boiled for 3 min prior to electrophoresis in 12.5% SDS-polyacrylamide gels. Following electrophoresis, gels were dried and exposed to Kodak XAR film. Proteins recovered from equal numbers of cells were compared, and all gels were stained with Coomassie blue to check that this condition had been met.

Preparation of polyribosomes. Polyribosomes were prepared by using a modification of the method described by Katze et al. (29). The medium was removed, and cells were scraped into ice-cold saline solution (10 mM Tris hydrochloride, pH 7.4, containing 150 mM NaCl) containing 50 μg of cycloheximide per ml and pelleted. Cells were then suspended in RSB (10 mM Tris hydrochloride, pH 7.4, containing 10 mM NaCl and 2 mM $MgCl_2$) containing 1% (vol/vol) Tween 40 and 0.5% (wt/vol) sodium deoxycholate and passed 3 times through a 25-gauge needle. The disrupted cells were centrifuged to remove nuclei, and the supernatants were layered onto 10 to 50% sucrose gradients containing 10 mM Tris hydrochloride, pH 7.4, 500 mM KCl, and 5 mM $MgCl_2$. Following centrifugation at 36,000 rpm for 2 h in an SW41 rotor, 1.1-ml fractions were collected from the bottom of each gradient and 10 μg of tRNA was added to each fraction.

Analysis of RNA. Fractions recovered from polyribosome gradients (see above) or the cytoplasmic fraction of infected cells were digested with 100 μg of proteinase K per ml in 20 mM Tris hydrochloride, pH 7.4, containing 3.5 M urea and 10 mM EDTA, and the RNA was purified by phenol-$CHCl_3$ extraction prior to ethanol precipitation. For Northern (RNA) blot analysis, equal quantities of RNA samples were glyoxalated and separated by electrophoresis in 0.9% agarose gels containing 10 mM phosphate buffer, pH 6.0. The glyoxalated RNA was then transferred electrophoretically to a nylon membrane (Biotrans) and UV cross-linked. Blots were hybridized with [^{32}P]oligo-labeled DNA probes (23) for the following adenovirus genes: E1B and pIX, E1B 22S cDNA (the generous gift of Nicki Harter); IVa$_2$, Ad2 *Hin*dIII fragment C (7.7 to 17.1 map units [m.u.]); L2, Ad2 *Hin*dIII fragment D (41 to 50.1 m.u.); L3 and L4, Ad2 *Hin*dIII fragment A (50.1 to 72.8 m.u.); L4, a *Sma*I to *Hin*dIII fragment (73.2 to 76.6 m.u.); and 28S rRNA, pA4 (26). Hybridization was in Church buffer (18) at 68°C, typically for 16 h. Blots were then washed twice in 0.1× SSPE (1× SSPE is 10 mM sodium phosphate, pH 7.4, containing 0.18 M NaCl and 0.1 mM EDTA) containing 0.1% (wt/vol) SDS at temperatures appropriate for each probe, dried, and exposed to Kodak XAR films in the presence of intensifying screens at −80°C. RNase protection analysis of E2 mRNA transcribed from the E2 early promoter was performed by the method of Melton et al. (39), using a probe made by T7 RNA polymerase transcription of an *Eco*RI-linearized plasmid containing the *Sma*I-*Hin*dIII fragment of Ad5 DNA, 73.2 to 76.6 m.u., cloned into pGEM4 (kindly provided by Tom Shenk).

To examine the accumulation of newly synthesized RNA in the cytoplasm, infected cells were labeled with 225 μCi of [^{3}H]uridine per ml in the presence of 14 μM unlabeled uridine at 18 h after infection at 39°C for increasing periods.

Cytoplasmic RNA was purified from each sample as described in the previous paragraph and hybridized for 2 days to 50 μg of linearized *Hin*dIII fragment D immobilized on nylon membrane filters under the conditions described above. Following washing, filters were dried and counted in Bray's solution, DNA side up.

Analysis of eIF-2α phosphorylation. Preparation of eIF-2 samples was by the methods of Scorne et al. (54) and O'Malley et al. (41). Infected cells were harvested into saline solution, pelleted, and suspended in 0.5 ml of 9.8 M urea–1% (vol/vol) Nonidet P-40–2% (vol/vol) mercaptoethanol–3% (vol/vol) 1:4 3-10:5-7 ampholines (LKB Instruments, Inc.). Samples were mixed at 30°C for 5 min, and the nuclei were pelleted by centrifugation at 1,000 × *g* for 1 min. Supernatants were frozen at −80°C until analysis. To separate the unphosphorylated and phosphorylated forms of eIF-2α, 50-μl portions containing 100 μg of bovine serum albumin per ml were analyzed by isoelectric focusing slab gel electrophoresis. eIF-2α was detected by Western immunoblotting by the method of Scorne et al., (54), using monoclonal antibody to eIF-2α, generously provided by E. Henshaw.

Immunoprecipitation. 293 cells on 60-mM dishes were labeled for 30 min at 18 h after infection with 500 μCi of [^{35}S]-Trans label (ICN Pharmaceuticals, Inc.). Cells were harvested and sonicated in 50 mM Tris hydrochloride, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, and 0.5% (vol/vol) Nonidet P-40. Portions of 2 × 10^5 cells were precleared with *Staphylococcus aureus* protein A for 1 h prior to incubation for 2 h on ice with excess antibody in 0.8 ml of isotonic buffer. After collection of antibody-antigen complexes with protein A, pellets were resuspended and repelleted four times in 50 mM Tris hydrochloride, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, and 0.05 or 0.5% (vol/vol) Nonidet P-40 in the case of E3 19- and E1B 55-kDa protein or E2A 72-kDa protein immunoprecipitation, respectively. The immunoprecipitates were then boiled for 3 min in GSB and analyzed by electrophoresis in 12.5% SDS-polyacrylamide gels followed by fluorography. The E1B 55, E2A 72, and the E3 19-kDa proteins were detected, using monoclonal antibody 58K-2A6 (50), monoclonal antibody β6 (47), and polyclonal rabbit antiserum raised against a C-terminal peptide of the E3 19-kDa protein (71), respectively.

RESULTS

The production of viral late proteins is impaired by the H5ts1 mutation. During a screen for adenovirus mutations that might alter the normal program of viral gene expression in productively infected cells, we observed that the labeling of viral late proteins was aberrant in H5ts1-infected 293 or HeLa cells maintained at a nonpermissive temperature. This phenotype is illustrated in Fig. 1, which shows typical results obtained when proteins were labeled with [^{35}S]methionine after increasing periods of Ad5 or H5ts1 infection of 293 cells. At 39°C, Ad5-infected cells exhibited the characteristic pattern of production of viral late proteins: such proteins, for example polypeptides II (hexon), III (penton base), and IV (fiber), were readily detected by 9 h after infection and by 18 h had become the predominant species labeled, as cellular protein synthesis was inhibited. By contrast, the labeling of these viral late proteins was significantly reduced in H5ts1-infected 293 cells at 39°C, despite comparable kinetics of inhibition of cellular proteins synthesis (Fig. 1, lanes 1 through 7). Similar low levels of labeling of viral late polypeptides were observed as late as 26 h after H5ts1 infection at 39°C, suggesting that this mutation does not

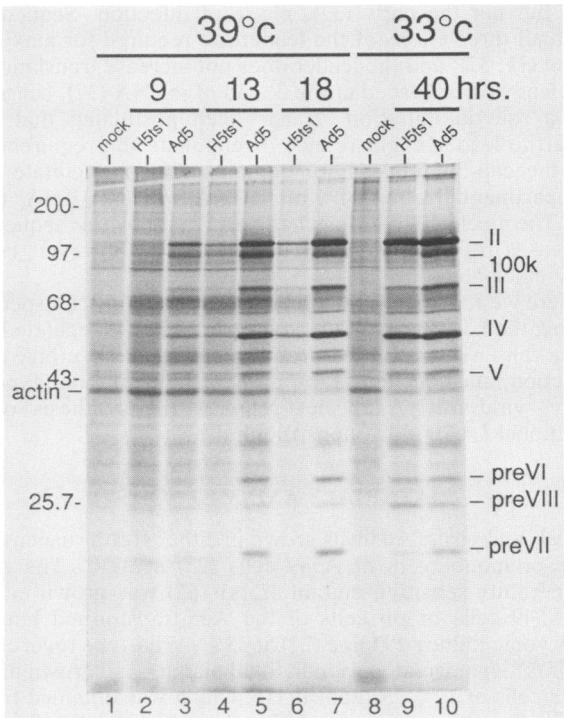

FIG. 1. Synthesis of viral late polypeptides in Ad5- and H5ts1-infected 293 cells. Infected cells were labeled with [^{35}S]methionine after the periods of infection at 39 or 33°C indicated, and total proteins were analyzed as described in Materials and Methods. The major viral polypeptides are listed at the right, and the positions to which prestained high-molecular-weight markers migrated are indicated at the left.

cause a delay in progression through the productive cycle. At 33°C, labeling of viral late polypeptides was comparable in Ad5- and H5ts1-infected cells (Fig. 1, lanes 9 and 10).

To determine whether synthesis of viral late polypeptides was defective in H5ts1-infected cells at nonpermissive temperatures or whether the viral proteins synthesized were unstable, pulse-chase experiments were performed. As illustrated in Fig. 2A, the viral proteins labeled with [^{35}S] methionine during a 30-min pulse-label in Ad5- and H5ts1-infected cells exhibited similar stabilities during a 3-h chase. Moreover, when proteins were labeled for so short a period that significant degradation would be unlikely, 2 min, substantially reduced levels of viral late polypeptides were still observed in H5ts1-infected cells (Fig. 2B). These results established that the H5ts1 mutation impairs the synthesis of viral late proteins in 293 cells.

The magnitude of this defect has been determined in five independent experiments by direct counting of polypeptide bands from gels like that shown in Fig. 1. Synthesis of polypeptides II, III, and IV, which could be readily quantitated in this manner, was reduced on average 11-, 9-, and 7-fold, respectively, in H5ts1-infected cells. The synthesis of smaller viral polypeptides, pVI, pVII, and pVIII, was inhibited to a similar extent, but quantitation was less reliable in these cases because the incorporation of [^{35}S]methionine into these protein was reduced to close to background levels in H5ts1-infected cells (e.g., Fig. 1, lane 6). Labeling of the L4 100-kDa protein, to which the H5ts1 mutation maps (see next section), was reduced some fivefold on average. The 100-kDa protein synthesized in H5ts1-infected cells appeared as stable as its wild-type counterpart (Fig. 2A). Thus,

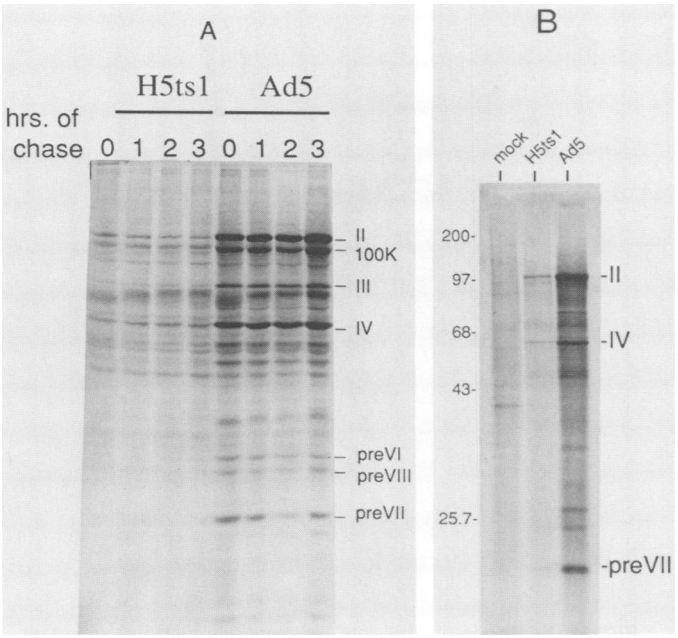

FIG. 2. Viral polypeptides synthesized in H5ts1- and Ad5-infected cells at 39°C exhibit similar stabilities. Ad5- or H5ts1-infected 293 cells were labeled at 18 h after infection for 30 min followed by a chase in the presence of excess unlabeled methionine for the periods indicated (A) or for 2 min (B), as described in Materials and Methods.

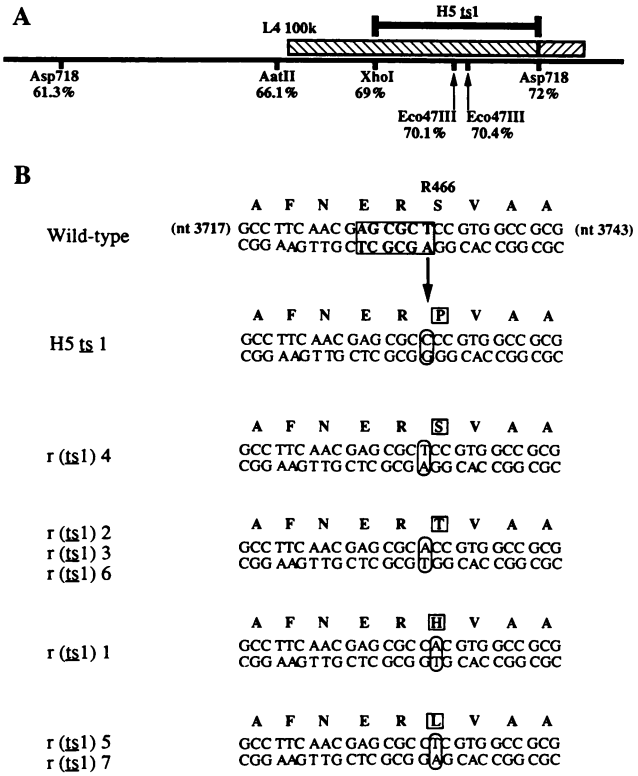

FIG. 3. Characterization of H5ts1 and reverse mutations. (A) Organization of the Ad5 genome in the region of the L4 100-kDa protein coding sequence. The viral DNA sequence is represented by a solid line, and the approximate map locations of relevant restriction endonuclease recognition sites are shown below this line. The crosshatched box shows the position of the coding sequence of the 100-kDa protein. The region of the left of the *Asp*718 site at 72 m.u. corresponds to the section of the Ad5 100-kDa protein that has been sequenced (35), while the region to the right of this site is the C-terminal region of the gene that has been determined in Ad2 (42). The solid bar above the box shows the marker rescue limits for the H5ts1 mutant, which extend from the *Xho*I site at 69 m.u. to the *Asp*718 site at 72 m.u. (B) Nucleotide sequence of the region of the 100-kDa protein coding sequence harboring the mutation in the H5ts1 virus. In the first part of the figure the region of the genome extending from nucleotides 3717 to 3743 of wild-type Ad5 is shown in double-stranded format (nucleotide numbers are from Kruijer et al. [35]). The *Eco*47III site at nucleotide 3727 (70.1 m.u.), the loss of which is responsible for the restriction-fragment-length polymorphism apparent in the DNA of the mutant and relevant revertant viruses, is shown boxed and shaded. The corresponding amino acid residues of the 100-kDa protein are shown above the DNA sequence as one-letter symbols. The mutated base pair in H5ts1 and the base pair changes that occur in transition from the mutant DNA sequence to the various revertant forms are shown boxed. The altered amino acids that are generated at residue 466 in the mutant and revertant forms of the protein are also boxed.

the decreased quantities of this polypeptide observed in H5ts1-infected cells must be the result of inefficient synthesis rather than a higher rate of turnover of the mutant protein. However, the H5ts1 mutation does alter the properties of the L4 100-kDa protein, which migrated slightly more slowly than its wild-type counterpart when examined directly (Fig. 1 and 2A) or after immunoprecipitation (data not shown).

We have examined a total of 10 mutant viruses carrying lesions mapped to the L4 100-kDa protein by the assay shown in Fig. 1. H5ts1 was significantly more defective than any other of these viruses (data not shown). However, the large degree of inhibition of viral protein synthesis observed in H5ts1-infected cells cannot be attributed to a dominant effect of the mutation, for normal levels of viral late proteins were synthesized in cells coinfected with H5ts1 and Ad5 (data not shown).

Characterization of the H5ts1 and reverse mutations. The H5ts1 mutant was originally isolated from wild-type Ad5 randomly mutagenized by hydroxylamine treatment (67). The mutation responsible for the temperature-sensitive phenotype of H5ts1 was mapped genetically and physically by recombinational analysis and intertypic recombination, respectively, to the region encoding the 100-kDa protein (49, 69). Subsequently, it was shown by marker rescue analysis to lie between m.u. 69.0 (*Xho*I site) and 72.0 (*Asp*718 Site) within the 100-kDa protein sequence (R. Galos and J. Williams, unpublished results). To locate the mutation precisely, the *Asp*718-*Aat*II fragment excised from the *Asp*718 E fragment of H5ts1 (Fig. 3) and the corresponding fragment from the Ad5 wild-type *Asp*718 E fragment were cloned into pUC18 and sequenced, using a series of primers spanning the rescue limits of H5ts1, as described in Materials and Methods. A single base pair change, comprising a T to C transition, was found at nucleotide 3732, 380 base pairs 5′ to the *Asp*I site at 72 m.u. in the viral genome (Fig. 3). This mutation alters residue 466 of the 100-kDa protein from serine (TCC) in the wild-type to proline (CCC) in the mutant protein.

The H5ts1 mutation introduces a restriction-fragment-length polymorphism, for it destroys one of the two *Eco*47III restriction sites within the *Asp*718 E fragment. These sites lie 257 and 383 base pairs upstream from the *Asp*718 site at 72 m.u. and produce a 126-base-pair fragment when Ad5 DNA

is cut with *Eco*47III (Fig. 3). The distal *Eco*47III site is destroyed by the H5ts1 mutation, and the 126-base-pair fragment is therefore missing when H5ts1 DNA is cut with this enzyme. This restriction-fragment-length polymorphism allowed a convenient and rapid screen of DNA from temperature-independent (ts^+) revertant isolates of H5ts1 to determine whether they were truly reverted at the original site of mutation or whether they possessed second-site mutations that suppressed the H5ts1 lesion.

A total of seven spontaneous, independent revertants were isolated and examined by the restriction-fragment-length-polymorphism screen, and all but one were found to contain the original H5ts1 mutation. To determine whether the second-site mutation of the first revertant obtained r(ts1)1, was intra- or extragenic, the *Sma*I E fragment (retaining the H5ts1 mutation) was cloned into pUC18 and used to rescue H5ts1. This fragment rescued H5ts1 as efficiently as did the equivalent Ad5 wild-type fragment (data not shown), indicating that the reversion must lie within the 100-kDa gene itself. Sequence analysis showed the r1 reversion to be a transversion of a C to an A at the second base of the CCC codon at position 466, resulting in replacement of proline by histidine (Fig. 3). Subsequent sequence analysis of the other revertants revealed that only one of the seven, r(ts1)4, was a true revertant, in which the first C of the codon was substituted by a T. The remaining five revertants also contained mutations within the first or second bases of the mutant codon, to replace proline by either threonine (3/5) or leucine (2/5). Because mutation at the third position of the codon would always generate proline, this set of revertants represents all but two, GCC (alanine) and CGC (arginine), of the six theoretically possible single base pair substitutions that could replace proline at codon 466. Although there is clearly no bias towards wild-type serine at position 466, threonine, structurally similar to serine, replaced proline in three out of the seven revertants.

Efficient synthesis of viral late polypeptides at 39°C was restored in 293 cells infected by each of the six revertants tested, and no differences could be detected among the true revertant [r(*ts*1)4] and those carrying second-site mutations (Fig. 4). We can, therefore, conclude that the temperature-sensitive H5ts1 phenotype illustrated in Fig. 1 is the result of a single mutation that replaces serine at codon 466 by proline in the L4 100-kDa protein-coding sequence. Moreover, any one of several mutations, which result in substitution of this proline residue by threonine, leucine, or histidine, appear to restore wild-type function as efficiently as reversion to the wild-type serine.

Synthesis of viral late mRNA in H5ts1-infected 293 cells. The defective protein synthesis displayed by cells infected with H5ts1 maintained at a nonpermissive temperature (Fig. 1 and 2) could be the result of either inefficient translation or a reduction in the concentration of viral late mRNA species available for translation. To distinguish between these possibilities, we examined the steady-state concentrations of cytoplasmic viral mRNA species in Ad5- and H5ts1-infected cells. Total cytoplasmic RNA, prepared from cells harvested 18 h after infection with either virus at 39°C, was analyzed by Northern blotting, as described in Materials and Methods. In the example shown in Fig. 5 with an L2-specific probe, which detects III, pVII, V, and pre-μmRNA species, identical steady-state concentrations of viral mRNA species were observed in H5ts1- and Ad5-infected cells. Similar results were obtained with probes specific for L3 (pVI and II), L4 (pVIII, 100, and 33 kDa), IVa_2 and pIX mRNAs (data not shown). To be sure that the metabolism of viral late

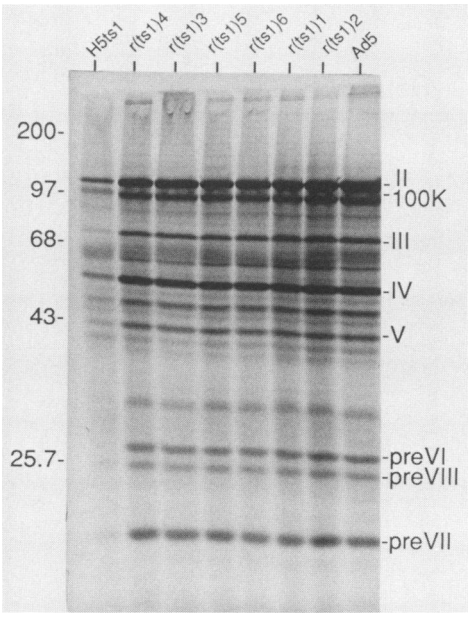

FIG. 4. Synthesis of viral late polypeptides in H5ts1 revertant virus-infected cells. Proteins synthesized in cells infected by the viruses listed were labeled at 18 h after infection at 39°C and analyzed as described in Materials and Methods. The major viral polypeptides are listed at the right, and the positions to which prestained high-molecular-weight markers migrated are indicated at the left.

mRNAs was unaffected by the mutation, we performed label accumulation experiments of the kind shown in Fig. 6. In these experiments, [^{3}H]uridine was added to 293 cells 18 h after infection. At 45-min intervals thereafter, total cytoplasmic RNA was isolated and hybridized to L2-specific DNA immobilized on nylon filters. The incorporation of the label into L2-specific RNA was linear in both Ad5- and H5ts1-infected 293 cells. Moreover, the rate of incorporation of label into L2-specific mRNA was the same in the two infected cell populations (Fig. 6). These results rule out the possibility that any significant change in the synthesis or half-lives of the RNA species occurred as a result of the

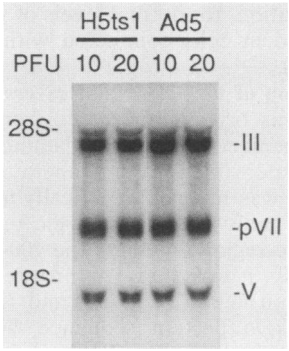

FIG. 5. Steady-state levels of viral L2 mRNAs in Ad5- and H5ts1-infected cells. Total cytoplasmic RNA prepared from Ad5- or H5ts1-infected cells at 18 h after infection at 39°C was analyzed by Northern blotting with a probe specific for L2 mRNAs, as described in Materials and Methods. The major L2 mRNAs are indicated at the right, and the positions to which 28S and 18S rRNA migrated are indicated at the left.

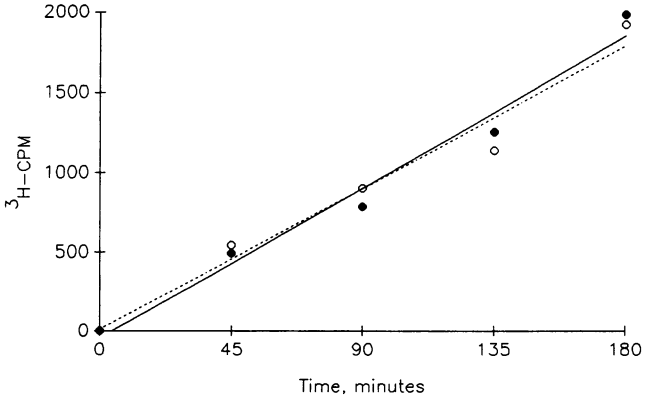

FIG. 6. Accumulation of newly synthesized L2 mRNA in the cytoplasm of Ad5- and H5ts1-infected cells. Ad5- or H5ts1-infected 293 cells were labeled with [^{3}H]uridine at 18 h after infection at 39°C. Cells were harvested at 45-min intervals, and the incorporation of label into L2 RNA isolated from Ad5 (closed circles)- and H5ts1 (open circles)-infected cells was determined as described in Materials and Methods. RNA prepared from uninfected 293 cells labeled for 180 min gave a background hybridization value of 230 cpm.

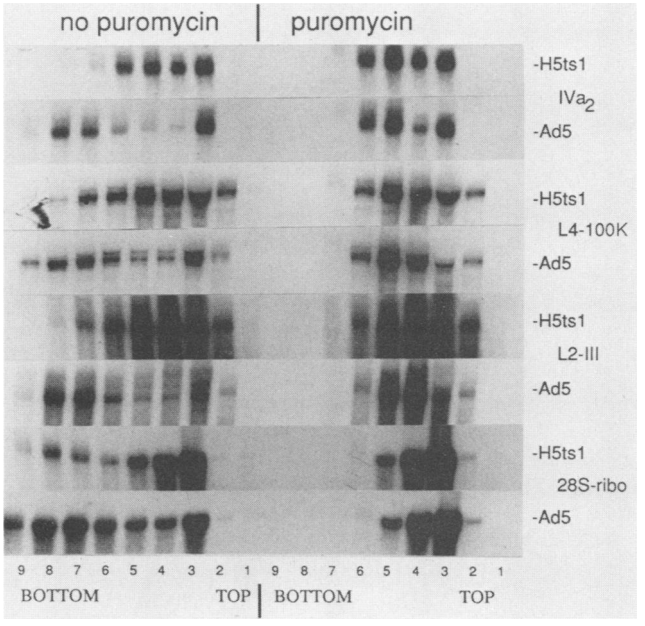

FIG. 7. Polyribosome association of viral late mRNA species in Ad5- and H5ts1-infected cells. At 18 h after infection at 39°C, polyribosomes were prepared and analyzed by sucrose gradient sedimentation as described in Materials and Methods. The positions in the polyribosome gradients of the RNA species listed at the right were determined by Northern blotting. In these experiments, extracts prepared from one 100-mm plate of Ad5- or H5ts1-infected cells were loaded onto gradients without normalization for cell number. The gradients shown in the right-hand column were from samples treated with 200 μM puromycin for 45 min immediately before cells were harvested.

H5ts1 mutation. Because the production and cytoplasmic accumulation of viral late mRNA species are normal in cells infected with H5ts1 (Fig. 5 and 6), the defect in synthesis of viral late polypeptides at 39°C (Fig. 1 and 2) must be translational.

Initiation of translation of viral late mRNAs is impaired by the H5ts1 mutation. In an attempt to characterize the translational defect displayed by H5ts1-infected cells at nonpermissive temperatures, we compared the distribution of viral late mRNA species among polyribosomes in Ad5- and H5ts1-infected cells. Cytoplasmic extracts were prepared 18 h after infection with either virus and sedimented in sucrose gradients (see Materials and Methods). RNA was purified from each fraction collected from the gradients and analyzed by Northern blotting. A second set of extracts was prepared, and analyzed in parallel, from infected cells treated with 200 μM puromycin for 45 min immediately prior to harvesting. This latter treatment induces polyribosome dissociation and release of mRNA (13, 40). Thus, the puromycin-treated samples provided markers for rRNA or mRNA species that were not actively engaged in translation. Figure 7 shows typical results, obtained with probes specific for the L2 protein III and the L4 100-kDa protein mRNAs, which contain the tripartite leader (7, 17), and the IVa_2 mRNA, which does not (16, 63). The distribution of 28S rRNA, which serves as a marker for the polyribosomes themselves, was also determined in each experiment and is illustrated in Fig. 7. Viral L2, L4, or IVa_2 mRNAs recovered from all samples that were treated with puromycin sedimented in the top half of the gradient, fractions 2 to 5 (Fig. 7, right-hand columns). Similarly, 28S rRNA was largely recovered in fractions 3 and 4 under these conditions, as expected. A large fraction of the viral mRNA species, or 28S rRNA, recovered from Ad5-infected cells that had not been exposed to the drug, sedimented to the bottom half of the gradient and was largely recovered in fractions 7 and 8 (Fig. 7), indicating efficient translation of these mRNAs, as expected by 18 h after infection (see introduction). A markedly different distribution of viral late mRNA species was observed in H5ts1-infected cells: only a small fraction of each mRNA species was recovered in fractions 7 and 8, and the majority was found nearer the top of the gradient (Fig. 7). The distributions of the IVa_2 and L2-III mRNA species observed in H5ts1-infected cells were similar to that induced by puromycin treatment of either Ad5- or H5ts1-infected cells: under both conditions, the mRNA species were concentrated in fractions 3 to 6 (Fig. 7). Differences in the polyribosome association in Ad5- compared with H5ts1-infected cells similar to those shown in Fig. 7 were observed for all other late mRNA species examined, a set that included the other L2, L3, L4, and protein IX mRNAs (data not shown, but see Fig. 8). Most of the 28S rRNA recovered from H5ts1-infected cells sedimented to fractions 3 to 5, the position at which 28S rRNA was observed in puromycin-treated cells (Fig. 7), indicating that the number of polyribosomes formed was significantly decreased in H5ts1-infected cells. These results confirm that viral late mRNA species are inefficiently translated in H5ts1-infected cells maintained at a nonpermissive temperature and indicate that initiation of translation is defective.

The shift towards the top of gradient in the distribution of 28S rRNA in H5ts1- compared with Ad5-infected cell samples (Fig. 7), which indicated that translation of cellular as well as viral late mRNAs was inhibited in H5ts1-infected cells, is consistent with the normal inhibition of host cell protein synthesis observed in mutant virus-infected cells (Fig. 1).

The H5ts1-mutation blocks the translation of adenoviral late, but not most early, mRNA species. We wished to determine whether translation of viral early mRNA species was also defective in H5ts1-infected cells at 39°C. Experiments like that shown in Fig. 7 were therefore performed at

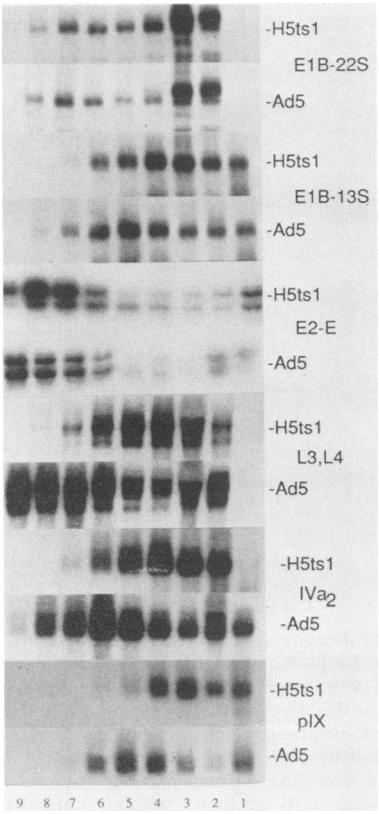

FIG. 8. Polyribosome association of viral early mRNA species in Ad5- and H5ts1-infected cells. The association of early mRNA species with polyribosomes in cells infected with Ad5 or H5ts1 for 15 h at 39°C was examined by Northern blotting or an RNAase protection assay as described in Materials and Methods and the legend to Fig. 7. The RNA probe used to detect E2A mRNA species spanned the early transcription initiation site but lacked the site near 72 m.u. used only during the late phase of infection. Thus, this probe detects only E2 early mRNA. The mRNA species analyzed are listed at the right: L3, L3 hexon and pVI mRNAs; L4, L4 100-kDa protein mRNA.

15 h after infection, when infected cells contained higher concentrations of early mRNA species (data not shown). Typical results obtained when the polyribosome associations of early and late mRNA species were compared in Ad5 and H5ts1-infected 293 cells at 39°C are shown in Fig. 8. The late mRNA species examined in this experiment, IVa_2, pIX, and L4 mRNAs, were again found nearer the top of the gradient in H5ts1- compared with Ad5-infected cell samples. By contrast, the polyribosome profiles of E1B 22S mRNA and E2A early mRNA were identical in wild-type and mutant virus-infected cells: a major fraction of both these early mRNA species was observed in the polyribosome region of the gradients (Fig. 8), indicating that their translation was not sensitive to the H5ts1 mutation. Similar results were obtained when E1A 13S and 12S mRNAs were examined (data not shown).

We also compared the synthesis of early proteins in Ad5- and H5ts1-infected cells at 13 h after infection. No differences in the synthesis of the E2A 72-kDa DNA-binding protein or the E3 19-kDa glycoprotein in Ad5- and H5ts1-infected cells could be detected when these proteins were immunoprecipitated, despite substantial inhibition of synthesis of late proteins in mutant virus-infected cells (Fig. 9). Nor was the synthesis of E1B 55-kDa protein or the steady-state levels of the E1A proteins, determined by Western blotting, decreased in H5ts1-infected cells (data not shown). The E1A and E1B mRNAs and proteins examined in these experiments are expressed from both integrated viral DNA (3, 27) and infecting viral genomes. However, this property cannot account for the insensitivity of their translation to the H5ts1 mutation, for identical results were observed when E2 and E3 proteins were examined. Thus, we can conclude from the results shown in Fig. 8 and 9 that the translation of typical viral early mRNA species during the late phase of adenovirus infection does not depend on the presence of a functional 100-kDa protein. However, translation of two mRNAs encoded by early transcription units, the E1B 13S and E1A 9S mRNA species, has been reproducibly observed to be more efficient in Ad5- than in H5ts1-infected cells (Fig. 8, rows 3 and 4; data not shown).

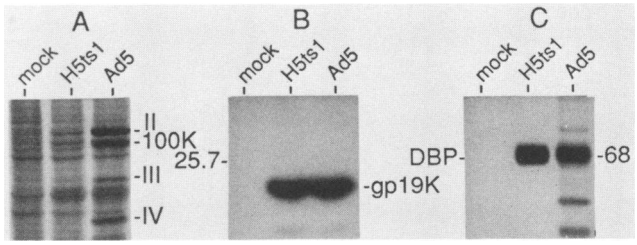

FIG. 9. Synthesis of viral early proteins during the late phase of infection in Ad5- and H5ts1-infected cells. Infected 293 cells were labeled with 500 μCi of [^{35}S]methionine per ml for 30 min at 13 h after infection at 39°C. Total proteins recovered are shown in panel A. The same samples were immunoprecipitated with monoclonal antibodies recognizing the E3 19-kDa glycoprotein (B) or the E2A 72-kDa DNA-binding protein (DBP) (C), as described in Materials and Methods.

The phosphorylation of eIF-2α is not altered by the H5ts1 mutation. It has been suggested that the phosphorylation of the α-subunit of eIF-2, which takes place to some extent in wild-type adenovirus-infected cells, is responsible for the inhibition of cellular protein synthesis that is characteristic of the late phase of adenovirus infection (41). To determine whether increased phosphorylation of this translation factor played any role in the defective initiation of translation of viral late mRNA species in H5ts1-infected cells, the phosphorylation of eIF-2α was examined. The unphosphorylated and phosphorylated forms of eIF-2α present in cells harvested 14, 18, or 22 h after infection at 39°C were separated by isoelectric focusing and assayed by Western blotting, as described in Materials and Methods. Cells infected by H5dl331, which produces no VA-RNA_I (60), were included as a positive control, for in the absence of this RNA, activated P1/eIF-2α kinase phosphorylates sufficient eIF-2α to inhibit protein synthesis (see introduction). In *dl*331-infected 293 cells, some 50% of the eIF-2α was phosphorylated by 22 h after infection (Fig. 10). By contrast, the phosphorylated species contributed no more than 15% of the total eIF-2α at any time examined in either Ad5- or H5ts1-infected cells (Fig. 10). The levels of eIF-2α phosphorylation we observed are somewhat lower than the values reported by O'Malley et al. (41), 25 and 90% in Ad5- and H5dl331-infected cells, respectively. These lower values may be the result of infecting a different line of 293 cells, for protein synthesis was not inhibited upon H5dl331 infection of the 293 cells used in these experiments to the extent reported by others (Fig. 11, lane 5). Be that as it may, the results shown

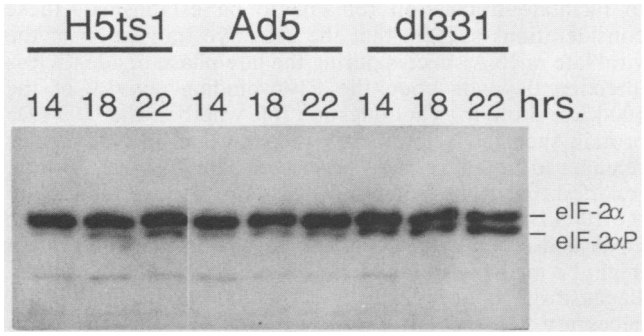

FIG. 10. Phosphorylation of eIF-2α in Ad5, *dl*331-, and H5ts1-infected cells. 293 cells were infected with Ad5, H5ts1, and *dl*331 for 14, 18, and 22 h at 39°C, and extracts were prepared as described in Materials and Methods. The relative concentrations of phosphorylated and unphosphorylated forms of eIF-2α were determined by isoelectric focusing slab gel electrophoresis and Western blotting.

in Fig. 10 suggest that altered phosphorylation of the α-subunit of translation initiation factor eIF-2 is not responsible for the inefficient initiation of translation induced by the H5ts1 mutation.

To confirm this conclusion, the ability of Ad5, H5ts1, and H5dl331 to direct synthesis of viral late proteins was also examined in a line of KB cells in which P1/eIF-2α kinase cannot be activated by adenovirus infection (32). At 18 h after infection at 39°C, the synthesis of viral late proteins was normal in H5dl331-infected KB cells but significantly reduced in 293 cells infected by this mutant virus (Fig. 11), as anticipated (32). By contrast, H5ts1 failed to support efficient synthesis of viral late proteins in either cell type (Fig. 11). Similar results were obtained with H5ts1-infected HeLa monolayer cells, in which inhibition of cellular protein synthesis was inefficient (data not shown). Thus, it is very unlikely that the inefficient inhibition of translation of late mRNA species characteristic of H5ts1-infected cells results from inactivation of eIF-2 by phosphorylation of its α-subunit.

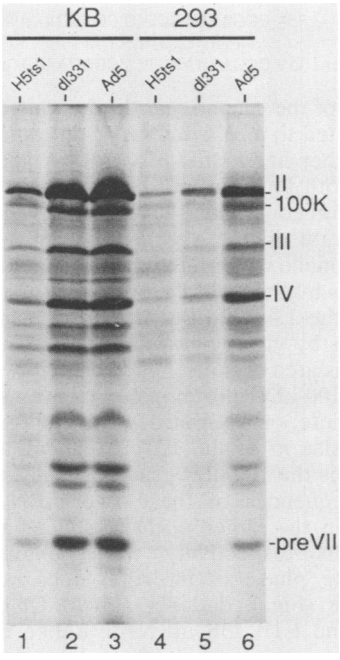

FIG. 11. Synthesis of viral late polypeptides in Ad5- H5ts1-, and *dl*331-infected KB and 293 cells. KB(A) or 293 (B) cells were infected with the viruses indicated for 18 h at 39°C, and the synthesis of viral late proteins was examined as described in the legend to Fig. 1.

DISCUSSION

The characterization of the phenotype displayed by H5ts1 reported here has established that the efficient translation of adenoviral late mRNA species requires the L4 100-kDa protein: the reduced quantities of the newly synthesized, viral late polypeptides accumulating in H5ts1- compared with Ad5-infected 293 cells could be attributed to neither an increased rate of protein turnover (Fig. 2) nor impaired production of viral late mRNA species (Fig. 5 and 6). Rather, the efficiency with which translation of the corresponding viral mRNA species was initiated was severely reduced in mutant virus-infected cells (Fig. 7 and 8). This translational defect can be unequivocally attributed to a single mutation mapping within the L4 100-kDa protein-encoding sequence, for true reversion, or any one of five second site mutations of the one transition identified in the H5ts1 100-kDa protein sequence (Fig. 3), restored the wild-type phenotype (Fig. 4).

The L4 100-kDa protein is specifically associated with hexon in group C adenovirus-infected cells (14, 24, 43, 44) and has been shown to play an essential role in hexon morphogenesis (15, 44). Indeed, the H5ts1 mutation blocks hexon trimerization (15). It seems unlikely that the translational role of the 100-kDa protein described here represents a secondary consequence of the failure of H5ts1-infected cells to produce hexon trimers: we have examined synthesis of viral late proteins in 293 cells infected by H5ts2, a hexon mutant (69) that is also unable to produce hexon trimers (15), and found a reduction of at most 50% (data not shown). In addition, the synthesis of viral late proteins is reduced by no more than 50% in cells infected by 100-kDa mutants H5ts3 and H5ts20 (data not shown), which are defective for trimerization of hexon (15). Finally, hexon trimerization takes place normally (15), but synthesis of viral late proteins (21, 33) is severely impaired in abortively infected monkey CV-1 cells in which the 100-kDa protein is largely mislocalized (15).

Although the 100-kDa protein is necessary for efficient synthesis of viral late proteins, it appears to play no role in the inhibition of cellular protein synthesis characteristic of adenovirus infection (Fig. 1). Several of the temperature-sensitive mutant viruses whose lesions have been mapped to the 100-kDa protein coding sequence that we have examined exhibited, albeit to a lesser degree, the inefficient synthesis of viral late proteins illustrated for H5ts1 in Fig. 1 (data not shown). All but one (H5ts17) also induced normal inhibition of cellular protein synthesis. This one exception proved to be a double mutant, whose single 100-kDa protein mutation imparted the typical phenotype shown in Fig. 1 when separated from the second mutation (B. Hayes, unpublished observations). Nor does translation of typical viral early mRNA species during the late phase of infection appear to depend on production of functional 100-kDa protein: neither labeling of the early proteins (Fig. 9) nor association of most of the early mRNA species with polyribosomes (Fig. 8) examined in these experiments was sensitive to the H5ts1 mutation. These phenotypes of H5ts1 indicate that the L4 100-kDa protein facilitates production of the viral late struc-

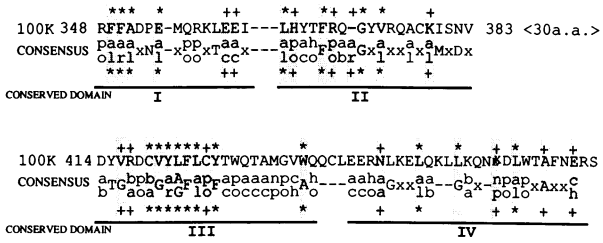

FIG. 12. RNA-binding protein consensus sequences in the L4 100-kDa protein. The consensus sequences of conserved domains I to IV of RNA-binding proteins is taken from Query et al. (46). po, Polar residue; al, branched-chain aliphatic residue (L, I, and V); ba, basic residue; ac, acidic residue; ho, hydrophobic residue; ch, charged residue; ar, aromotic residue; np, nonpolar residue; ab, amides, acids, or bases (EQDNKR); aa, amides or acids (EQDN); capital letters, standard amino acid abbreviations; and x, unassigned. The highly conserved positions are shaded, and regions marked by a star are more conserved than those marked by a plus. Sequences of the Ad2 100-kDa protein that conform to this consensus are shown above, aligned to maximize matches. The 100-kDa protein differs from the RNA-binding protein sequences from which the consensus was derived by the presence of 30 amino acids between conserved domains II and III.

tural and nonstructural proteins essential to assembly of progeny virions by specifically increasing the efficiency with which translation of their mRNAs is initiated. It is, moreover, clear that the 100-kDa protein does not facilitate translation of viral late mRNA species simply by circumventing the inhibitory consequences of activation of P1/eIF-2α kinase, recently suggested to be implicated in the inhibition of cellular protein synthesis (41); this protein is required for efficient synthesis of viral late proteins even when infection does not activate P1/eIF-2α kinase (Fig. 11). Under these conditions, *dl*331, which lacks the VA-RNAI promoter (60), directs the synthesis of wild-type levels of viral late proteins (Fig. 11). Thus, the ability of the 100-kDa protein to stimulate translation of viral late mRNA species cannot depend on the presence of VA-RNA$_I$ in infected cells.

It is well established that the 100-kDa protein binds to cytoplasmic mRNA. Adam and Dreyfuss (1) used UV-light-induced cross-linking to demonstrate that the 100-kDa protein makes direct contact with mRNA, substantiating several early reports of association of the protein with polyribosomal viral mRNA (36, 58, 59, 64). An extensive RNA recognition motif common to RNA-binding proteins has been recently identified and, in the case of the UI snRNP 70-kDa protein, shown to lie within the experimentally defined RNA binding region of the protein (46). The common RNA recognition region comprises four subdomains (I to IV), one of which (III) corresponds to the highly conserved RNA-binding protein octamer sequence previously recognized by Adam et al. (2). Sequences that conform to each of the subdomain consensus sequences can be found in the adenovirus L4 100-kDa protein (Fig. 12). In this protein, subdomain IV is entirely included in a segment, amino acids 437 to 478, that is predicted to form a continuous α-helix. Introduction of a proline residue at position 466, as in the H5ts1 100-kDa protein (Fig. 3), would disrupt this helix and thus might introduce a significant, temperature-dependent change in the geometry of the putative RNA recognition region of the protein. Each of the amino acids observed at codon 466 in revertant 100-kDa proteins (Fig. 3) would be compatible with helix formation. Thus, although a link between the ability of the 100-kDa protein to bind RNA and to facilitate translation remains to be established, these considerations suggest that the selective translation of the viral late mRNA species during the late phase of adenovirus infection depends upon the RNA-binding activity of the 100-kDa protein. The question of whether the 100-kDa protein specifically interacts with viral late mRNA species remains to be addressed. Adam and Dreyfuss (1), in fact, reported that RNA isolated by virtue of its cross-linking to the 100-kDa protein hybridized to both adenoviral genomic DNA and HeLa cell cDNA and suggested that the protein might be involved in inhibition of cellular protein synthesis. Because our data indicate that the 100-kDa protein is not necessary for the latter process (e.g., Fig. 1), it seems possible that the L4 100-kDa protein, although capable of binding to both viral and cellular mRNAs (1), possesses a greater affinity for the former. Such a difference in affinity, which it should be possible to detect by using more sensitive assays, would readily account for the restricted translational role of the 100-kDa protein. If the 100-kDa protein were a direct effector of translation, its specific binding to viral late mRNAs could facilitate their translation by either increasing the efficiency of some normal step in the initiation sequence or bypassing, or reducing, the requirement for one or more cellular initiation factors that had become limiting as a result of adenovirus infection.

One obvious candidate for specific recognition by the L4 100-kDa protein would be the tripartite leader (see introduction). However, efficient translation of both IVa$_2$ and IX mRNAs, which lack the tripartite leader (see references 24 and 56), also requires the 100 kDa protein (Fig. 7 and 8). The presence in these mRNAs of primary sequence or structural features related to those of the tripartite leader would provide a simple resolution to this apparent problem. Indeed, the IVa$_2$ 5′ untranslated sequence shares regions of homology with the tripartite leader sequence, the most extensive of which is the sequence

```
IVa2          34 CGGAACACCGCTCGAGGACAGGCCTCTCA 62
                 ||||| ||| |||||| | |||| ||| |
Tripartite leader 159 CGGAAAACCTCTCGAGAA-AGGCGTCTAA 186
```

Other regions of the second and third exons of the tripartite leader are related to a pIX mRNA 5′ untranslated sequence (9). On the other hand, this pIX mRNA sequence closely matches, in both location and primary sequence, a 5′ untranslated region consensus sequence derived from a survey of 699 vertebrate mRNA species (34), properties that are difficult to reconcile with preferential recognition of viral late mRNA species by the 100-kDa protein. It therefore appears unlikely that the L4 100-kDa protein recognizes viral late mRNA species by virtue of a primary sequence common to their 5′ untranslated regions.

While the 100-kDa protein might recognize sequences located elsewhere, or RNA structural features, the observation that translation of the E1A 9S and E1B 13S mRNA species requires the 100-kDa protein suggests an alternative model. All sequences of these two mRNA species are included within the larger mRNAs spliced from E1A and E1B transcripts, respectively, whose efficient translation during the late phase of infection does not require the 100-kDa protein (Fig. 8; data not shown). On the other hand, the E1A 9S and E1B 13S mRNAs, unlike the other early mRNA species examined in these experiments, do not accumulate to significant levels in the cytoplasm until the late phase of infection (e.g., see references 11, 57 and 70). It is therefore possible that the precursors to E1A 9S and E1B 13S mRNAs, like late mRNA species encoded by the major

late and pIX transcription units (19, 61), are transcribed from replicated, rather than parental, viral DNA molecules and that such late-phase-specific templates determine the preferential production and utilization of cytoplasmic viral mRNA species during the late phase of adenovirus infection. In such a "gating" (12) model, the primary role of the L4 100-kDa protein would be to direct viral late mRNA species to, or maintain them in, a physical or biochemical compartment in the cytoplasm in which their translation were facilitated. Similarly, it has been suggested that the efficient translation of heat shock proteins in cells exposed to inducers of the stress response might be related to the preferential synthesis of these mRNA species (62). Because the mRNA species whose efficient translation depends on the L4 100 kDa protein can be most readily cataloged in terms of their pattern of expression in infected cells, this kind of position effect model presently appears to provide a parsimonious explanation of the translational function of this viral protein. Clearly, however, additional experiments will be required to establish the mechanism by which the 100-kDa protein induces efficient translation of a specific set of viral mRNA species during the late phase of infection and to determine how the activity of this protein is integrated with the other circuits that govern translation in adenovirus-infected cells.

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