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The Function(s) Provided by the Adenovirus-Specified, DNA-Binding Protein Required for Viral Late Gene Expression Is Independent of the Role of the Protein in Viral DNA Replication

STEPHEN A. RICE AND DANIEL F. KLESSIG*

Department of Cellular, Viral and Molecular Biology, School of Medicine, University of Utah, Salt Lake City, Utah 84132

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The adenovirus type 2 (Ad2) host range mutant Ad2*hr*400 grows efficiently in cultured monkey cells at 37°C, but is cold sensitive for plaque formation and late gene expression at 32.5°C. After nitrous acid mutagenesis of an Ad2*hr*400 stock, cold-resistant variants were selected in CV_1 monkey cells at 32.5°C. One such variant, Ad2*ts*400, was also temperature sensitive (*ts*) for growth in both CV_1 and HeLa cells. Marker rescue analysis has been used to show that the two phenotypes, cold resistant and temperature sensitive, are due to two independent mutations, each of which resides in a different segment of the gene encoding the 72-kilodalton DNA binding protein (DBP). The cold-resistant mutation (map coordinates 63.6 to 66) is a host range alteration that enhances the ability of the virus to express late genes and grow productively in monkey cells at 32.5°C. The temperature-sensitive mutation is in the same complementation group and maps to the same segment of the DBP gene (map coordinates 61.3 to 63.6) as the well-characterized DBP mutant Ad5*ts*125. Like Ad5*ts*125, Ad2*ts*400 is unable to replicate viral DNA or to properly shut off early mRNA expression at the nonpermissive temperature. Two sets of experiments with Ad2*ts*400 suggest that DBP contains separate functional domains. First, when CV_1 cells are coinfected at the nonpermissive temperature with Ad2 plus Ad2*ts*400 (Ad2 allows DNA replication and entry into, but not completion of, the late phase of infection), normal late gene expression and productive growth occur. Second, temperature shift experiments show that, although DNA replication is severely restricted at the nonpermissive temperature in *ts*400-infected monkey cells, late gene expression occurs normally. These results indicate that the DBP activity required for normal late gene expression in monkey cells is functional even when the DBP's DNA replication activity is disrupted.

The 72-kilodalton DNA-binding protein (DBP), encoded in early region 2A of the adenovirus genome, is a phosphoprotein with an avid affinity for single-stranded DNA (66). Unlike most other adenovirus proteins, DBP is expressed at substantial levels both early and late during the viral life cycle (23).

Analysis of two general classes of DBP mutants has indicated that DBP performs several seemingly diverse functions in the infected cell. The first class of mutants is typified by the temperature-sensitive adenovirus type 5 (Ad5) mutant Ad5*ts*125 (18) and suggests a role for DBP in viral DNA replication, early gene expression, and cellular transformation. Ad5*ts*125 synthesizes a DBP that is thermolabile in its capacity to bind to single-stranded DNA in vitro (67). At the nonpermissive temperature, Ad5*ts*125 is unable to replicate viral DNA both in vivo (18) and in an in vitro system (36). In vivo the *ts*125 defect appears to affect both the initiation (68) and chain elongation (36, 69) steps of viral DNA synthesis, although more recent studies have shown that in vitro DBP is only required for chain elongation (14, 25). In addition, the normal turnoff of early mRNA expression does not occur at the nonpermissive temperature in cells infected with Ad5*ts*125 (10, 11). This is not simply due to the inhibition of viral DNA synthesis, since inhibition of DNA synthesis by drugs or by infection with DNA-negative mutants in other early genes does not have the same effect. The negative regulation of early mRNA expression mediated by the wild-type (WT) DBP appears to act at the level of transcription for early region 4 (33, 50) and at the level of mRNA turnover for early regions 1A and 1B (7). Furthermore, Ad5*ts*125 transforms cultured rat cells with greater efficiency than WT virus, even at permissive temperatures (28, 70).

A second class of DBP mutants that grow productively in monkey cells (2, 40, 43) suggests that DBP also plays a role in late gene expression. WT adenovirus does not grow in cultured monkey cells even though virus adsorption (20), early gene expression (4, 20, 24), and DNA replication (24, 34, 57) all appear normal. Synthesis of most of the late viral proteins, however, is greatly diminished (8, 24, 41). This reduction can in most cases be accounted for by a reduction in the steady-state levels of encoding mRNAs (19, 41). This is not the case for the capsid protein fiber, however. Fiber protein synthesis is reduced more than 100-fold relative to a productive infection, whereas fiber mRNA levels are reduced only 5- to 10-fold (5, 41, 42). The block to fiber expression may involve both abberant processing (42) and translation (5) of the fiber mRNA. Host range (*hr*) mutants of adenovirus (Ad2*hr*400 to 403, Ad5*hr*404, and $Ad2^+$ND3*hr*600 to 603) have been selected by their ability to grow in monkey cells, and the mutations responsible have been localized to the DBP gene (3, 43, 44). Thus, an altered DBP can in some way allow the normal expression of late genes in monkey cells. A similar function is carried by the carboxyl-terminal portion of simian virus 40 (SV40) large T antigen, since coinfection of monkey cells with WT adenovirus plus SV40 allows normal late gene expression (56), as does infection with Ad2-SV40 hybrid viruses that contain only the SV40 sequences encoding the C-terminal segment of T antigen (21, 31, 47).

Physical mapping of the two classes of DBP mutations suggests that the protein contains functionally distinct do-

* Corresponding author.

mains (44). Both Ad5*ts*125 and the phenotypically similar mutant Ad5*ts*107 map to the carboxyl-terminal half of the DBP gene (coordinates 60.2 to 63.6 on the adenovirus map), whereas the *hr* mutants map to the amino-terminal portion of the gene (coordinates 63.6 to 66.0). Biochemical studies also suggest the existence of two domains. Mild treatment of purified DBP with chymotrypsin generates two products: a carboxyl-terminal 44-kd fragment and a 26-kd amino-terminal fragment (39, 60). The 44-kd polypeptide is similar in size to the C-terminal breakdown product of DBP often observed in extracts from infected cells (59, 66). The purified 44-kd fragment retains the DNA binding activity of DBP (39) and can complement an in vitro DNA-synthesizing system that is dependent upon exogenous DBP (6). The 26-kd N-terminal polypeptide has no DNA binding activity, but contains most of DBP's 9 to 11 phosphate residues (39).

Here we describe a novel adenovirus mutant, Ad2*ts*400, which contains DBP mutations of both types. As expected, the temperature-sensitive mutation that affects DNA replication and early gene expression maps in the C-terminal portion of the gene, whereas the host range alterations map in the N-terminal portion. Analysis of Ad2*ts*400 provides further evidence that the adenovirus DBP contains separate functional domains.

MATERIALS AND METHODS

Cells, viruses, and infections. CV_1 and CV_c cells, established lines of African green monkey kidney cells, were obtained from J. Mertz and P. Tegtmeyer, respectively. The human 293 and HeLa cell lines were obtained from J. F. Williams. Monolayer cultures of CV_c, 293, and HeLa cells were cultivated in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% calf serum (Irvine Scientific), 100 μg of streptomycin per ml, and 100 μg of penicillin per ml. For CV_1 cultivation, 5% calf serum and 2% fetal calf serum (Flow Laboratories, Inc.) were used instead of 10% calf serum. Suspension cultures of HeLa cells were grown in Eagle F13 medium (GIBCO).

Ad2 and Ad5 were originally obtained from U. Pettersson and J. F. Williams, respectively. Ad2*hr*400 and Ad2*hr*403 were isolated by D. F. Klessig (40, 43). All of the above virus were propagated in suspension cultures of HeLa cells. Ad5*ts*125, Ad5*ts*107, and Ad5*dl*434 were obtained from J. F. Williams, C. S. H. Young, and D. Solnick, respectively. Ad5*ts*125 and Ad5*ts*107 were propagated in monolayers of HeLa cells at 32.5°C, and Ad5*dl*434 was propagated in 293 monolayers at 37°C. SV40 strain 776 was obtained from J. Sambrook and prepared as previously described (61). Plaque assays were performed as described previously (31), except that $MgCl_2$ was omitted from titrations on 293 and CV_1 cells.

Confluent monolayers of CV_1, CV_c, or HeLa cells were infected at a multiplicity of 20 PFU per cell unless otherwise indicated. After adsorption for 75 min at 37°C in phosphate-buffered saline, the cells were washed, overlaid with medium, and incubated at the appropriate temperature.

Analysis of viral protein synthesis. The synthesis of viral proteins in vivo was assayed by labeling cell monolayers with 40 μCi of [^{35}S]methionine per ml and analyzing the products by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (41). Immunoprecipitation of the fiber protein from infected cell lysates was performed as previously described (5).

Analysis of viral DNA synthesis. Viral DNA synthesis was analyzed by labeling cell monolayers (60 mm) with 50 μCi of [^{3}H]thymidine (Amersham Corp.) per ml for 1 or 2.5 h. Viral DNA was extracted by a modified Hirt extraction (35). The labeled cell monolayers were washed once with phosphate-buffered saline and 3.2 ml of 10 mM Tris (pH 7.9)–10 mM EDTA–0.6% SDS–0.5 mg of pronase per ml was added to each. After incubation for 2 h at 37°C, 0.8 ml of 5 M NaCl was added dropwise to each plate. The viscous cell lysates were carefully poured into SW60 centrifuge tubes, incubated for 4 to 16 h at 4°C, and spun at 25,000 rpm, 4°C, for 1 h in an SW60 rotor. The supernatants were extracted once with phenol and once with chloroform-isoamyl alcohol (24:1) and precipitated with 2.5 volumes of ethanol.

[^{3}H]thymidine incorporation into adenovirus DNA was assayed in two ways. In the first method, equal fractions of each DNA preparation were digested with a restriction enzyme and fractionated by electrophoresis on a horizontal 1.2% agarose gel in 36 mM Tris–30 mM NaH_2PO_4–1 mM EDTA. After staining of the gel with 1 μg of ethidium bromide per ml and photography under UV illumination, the gel was soaked in En^{3}Hance (New England Nuclear Corp.) for 12 h and then in 5% trichloroacetic acid for 12 h. The gel was dried and exposed to XR-1 film with intensifying screens. In the second method, viral DNA synthesis was assayed by hybridizing equal fractions of each DNA preparation to Ad2 DNA immobilized on nitrocellulose filters (17). Before hybridization, the DNA samples were boiled for 5 min in 200 mM NaOH to denature DNA and degrade contaminating RNA. After hybridization at 42°C for 72 h in 50% formamide–0.4 M NaCl–0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 8.0)–5 mM EDTA–0.2% SDS–5 × Denhardt solution (1× Denhardt solution is 0.2% [wt/vol] bovine serum albumin, Ficoll [molecular weight, 400,000], and polyvinyl pyrrolidone)–100 μg of denatured salmon sperm DNA per ml, the filters were washed twice in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate)–0.1% SDS at room temperature, once in 1× SSC–0.1% SDS at 52°C, and twice in 0.1× SSC–0.1% SDS at 52°C. The dried filters were counted in 3 ml of Econofluor (New England Nuclear) in a liquid scintillation counter. The supernatants from the first hybridization were boiled for 5 min and hybridized to new filters. The amount of hybridization in the second round was less than 50% of the amount obtained in the first. [^{3}H]thymidine-labeled DNA from mock-infected cells was used to determine the background level of hybridization. The values given below (see Fig. 8a) are the sums of both hybridizations after subtraction of the backgrounds.

Physical mapping of temperature-sensitive and cold-resistant mutations. The DNA-terminal protein complex was prepared from Ad2 and Ad2*ts*400 by the method of Chinnadurai et al. (15), dialyzed extensively in 10 mM Tris (pH 7.8)–1 mM EDTA–1 mM β-mercaptoethanol, and stored at 4°C. Adenovirus genomic DNA was prepared by the method of Pettersson and Sambrook (54). Restriction enzyme-generated fragments of genomic DNA were fractionated on 40- by 20- by 0.6-cm horizontal 1% agarose gels in TBE buffer (90 mM Tris-borate, 2.5 mM EDTA, pH 8.3) by electrophoresis at a potential of 1 to 1.5 V/cm for 36 to 48 h. The separated fragments were eluted from the gel by electrophoresis as previously described (43). The positions at which restriction enzymes cleave the DNA of Ad2 have been taken from Tooze (65).

DNA infections (transfections) into 293 cells were performed by the method of Graham and van der Eb (30) and Stow et al. (63) and have been described previously (43, 44). The transfected 293 cells were incubated at 37°C for 4 to 5 h before glycerol treatment to boost transfection efficiency (26, 43).

For mapping of the Ad2*ts*400 temperature-sensitive muta-

tion, 293 cells were transfected with 0.5 μg of Ad2*ts*400 DNA-terminal protein complex per plate plus 4 or 8 molar equivalents of an individual restriction fragment derived from non-temperature-sensitive virus (Ad2, Ad2*hr*400, or Ad2*hr*403). After the glycerol treatment, the cells were incubated at 32.5°C. Progeny virus were released from the cells 4 days after transfection by freeze-thawing and sonication. One-tenth of each cell lysate was used to infect fresh monolayers of HeLa cells. After 72 h at 39.5°C, progeny virus were titrated on HeLa cells at 39.5°C to determine for each cross the number of virus that had acquired the ability to form plaques at 39.5°C. In one experiment, a second passage in HeLa cells at 39.5°C was done before titration.

For mapping of the Ad2*ts*400 cold-resistant mutation, 293 cells were transfected with 0.5 μg of Ad2 DNA-terminal protein complex per plate plus 10 or 20 molar equivalents of an individual restriction fragment derived from Ad2*ts*400 or 5 molar equivalents of unfractionated fragments derived from Ad2*hr*400 or Ad2*ts*400. The transfected cells were incubated at 32.5°C for 4 days. After release of progeny virus, 0.1 of the cell lysate was used to infect fresh monolayers of CV_c cells. After 4 days at 32.5°C, progeny virus were titrated on CV_1 cells at 32.5°C. Plaques were counted on day 18 to ensure that only virus with the cold-resistant phenotype were scored.

RNA preparation and analysis. Suspension cultures of HeLa cells (5×10^5 cells per ml) were infected with virus at a multiplicity of 100 at 41°C in the presence of 25 μg of cytosine arabinoside (araC) per ml to block DNA replication. araC was supplemented at 9 h postinfection (p.i.). At 6, 12, and 18 h p.i., approximately 2.5×10^7 cells were removed from each culture. Cytoplasmic RNA was prepared from these cells by phenol and chloroform extraction of the 0.5% Nonidet P-40 supernatant fractions followed by ethanol precipitation (41). Polyadenylated RNA was selected by passage over oligodeoxythymidylic acid-cellulose (type 3; Collaborative Research Inc.).

Polyadenylated RNA selected from 100 μg of each cytoplasmic RNA preparation was fractionated for 5 h at 70 V on a 14- by 16- by 0.4-cm 1.2% agarose gel containing 10 mM methyl mercury hydroxide. *Hin*dIII restriction fragments of Ad2 DNA were coelectrophoresed as size standards. The gel was stained with 0.5 μg of ethidium bromide per ml and examined under UV illumination to ensure that approximately equal amounts of RNA had been loaded in each lane. The RNA was transferred to diazobenzyloxymethyl-cellulose paper, and the diazobenzyloxymethyl paper was subsequently hybridized with radioactively labeled probes specific for each adenovirus early region as previously described (5).

All of the Ad2-specific probes used for hybridization were cloned fragments of Ad2 DNA inserted into a pBR322 plasmid. The *Hin*dIII-F (coordinates 89.5 to 97.1; early region 4) and *Hin*dIII-I (coordinates 31.5 to 37.3; late family 1) clones were obtained from S. L. Hu, and the *Bal*I-L clone (coordinates 6.0 to 7.7; early region 1B) was obtained from S. Berget. HEB4 (coordinates 0.8 to 4.5), a clone of a *Bal*I-*Hpa*I double digest fragment, was obtained from D. Solnick and was used as the probe for early region 1A. *Eco*RI-B (coordinates 58.5 to 70.7) and *Eco*RI-D (coordinates 75.9 to 84.0) clones were used as probes for early regions 2A and 3, respectively. Purified Ad5 *Eco*RI fragment C (coordinates 75.9 to 84.0) was used as the Ad5-specific probe for early region 3. The DNA used for probes was labeled by nick translation with [^{32}P]dCTP to a specific activity of 1×10^8 to 3×10^8 cpm/μg.

The autoradiographs shown in the text and those used for densitometry were obtained without intensifying screens so that band intensity would be proportional to the radioactivity on the blot. Densitometer tracings of the autoradiographs were performed with a Joyce-Loebl model 3CS microdensitometer, and areas under the peaks were determined with a Numonic Corp. model 1224 electronic graphics calculator.

RESULTS

Derivation of Ad2*ts*400. Ad2*hr*400 is a host range derivative of adenovirus that has acquired the ability to grow in CV_1 cells, an established line of African green monkey kidney cells, at 37°C (40). The mutation responsible for this phenotype is located in the gene encoding the 72-kd DBP (43). Ad2*hr*400, however, is partially cold sensitive for growth in CV_1 cells at 32.5°C. Although Ad2*hr*400 forms plaques on CV_1 cells with approximately equal efficiency at 32.5 and 37°C, the plaques at 32.5°C appear only after very long incubation and are minute in comparisons to those produced by the Ad2-SV40 hybrid virus Ad2'ND1. We suspected that the cold-sensitive phenotype of Ad2*hr*400 was related to the monkey cell block to late gene expression since Ad2*hr*400 was not cold sensitive for plaque formation on human (HeLa) cells. If this is true, then selection of cold-resistant variants might uncover new alterations in the DBP gene or in other genes whose products interact with monkey cell components to allow late gene expression. Furthermore, we anticipated that some cold-resistant variants selected for better growth in monkey cells at low temperature might simultaneously acquire a temperature-sensitive phenotype. Jarvik and Botstein (38) have observed this type of simultaneous switch from cold sensitivity to cold resistance and temperature sensitivity in bacteriophage P22.

A high-titer stock of Ad2*hr*400 was mutagenized with nitrous acid for 2 min as described previously (40). Portions of this mutagenized stock were used to infect CV_1 monolayers, and the infections were allowed to proceed at 32.5°C for 68 to 72 h before harvesting. The cell lysates were passaged twice more on CV_1 cells for 68 to 72 h at 32.5°C. Since 68 to 72 h approximates the minimum time required for WT Ad2 to complete an infectious cycle at this temperature, variants of Ad2*hr*400 that grow faster or with higher burst sizes (or both) are enriched in this scheme. After the third passage the cell lysates were titrated on CV_1 cells at 32.5 and 39.5°C, monitoring the total number of plaques appearing at the different temperatures as well as the rate of appearance of plaques at 32.5°C. Several of the independently passaged lysates yielded plaques that appeared sooner at 32.5°C than did Ad2*hr*400 plaques and were substantially larger. One such lysate also appeared to harbor a temperature-sensitive virus since the titer at 39.5°C was significantly less than the titer at 32.5°C. A virus from this stock was plaque purified three times on CV_1 cells at 32.5°C. High-titer stocks of this variant, Ad2*ts*400, were prepared by growth of the virus on either HeLa or CV_1 monolayers at 32.5°C.

Growth properties of Ad2*ts*400. The plaque-purified stock of Ad2*ts*400 was initially characterized by plaque assay on both HeLa and CV_1 cells at 32.5 and 39.5°C. As expected, Ad2*ts*400 plaques appeared sooner than did Ad2*hr*400 plaques at 32.5°C on CV_1 cells. In Ad2*ts*400 titrations, 50% of the plaques that eventually appeared on CV_1 cells at 32.5°C were visible by day 19, whereas in Ad2*hr*400 titrations 30 days were required to reach the 50% level. Furthermore, Ad2*ts*400 formed plaques on CV_1 monolayers at 32.5°C that were substantially larger than Ad2*hr*400 plaques (Fig. 1). Both viruses, however, formed plaques with similar

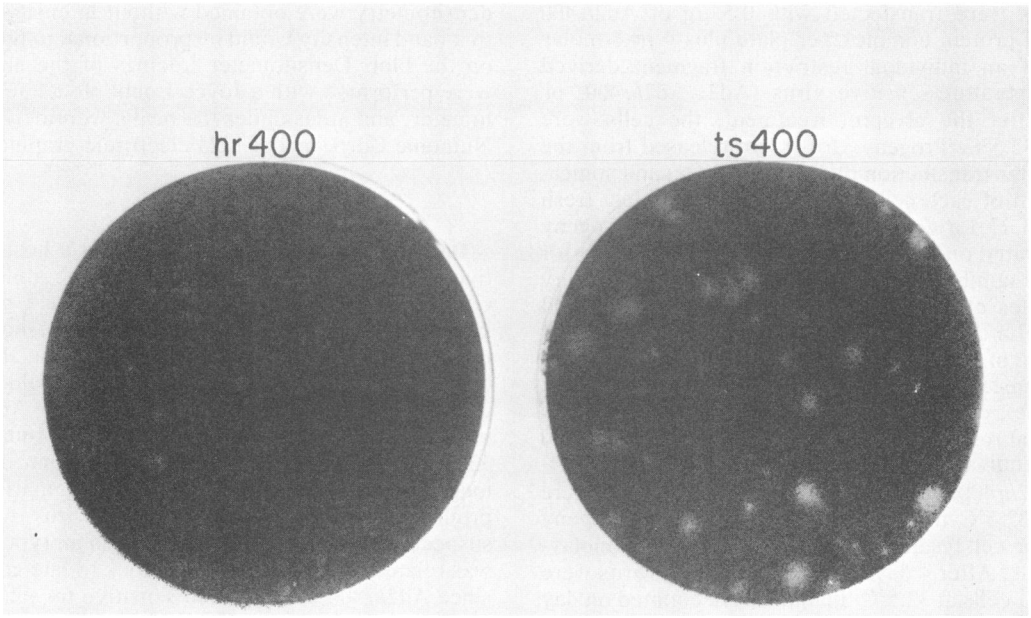

FIG. 1. Viral plaque assay on CV_1 monolayers at 32.5°C. Virus stocks were titrated as described by Grodzicker et al. (31). The cell monolayers were stained on day 18 with 0.01% neutral red, and the photograph shown was taken on day 25.

kinetics and morphology when titrated on HeLa cells at 32.5°C. In addition to its cold-resistant phenotype, Ad2*ts*400 appeared to be temperature sensitive, since it did not form plaques on either HeLa or CV_1 cells at 39.5°C, although the monolayers were killed at very low dilutions of virus.

To define more carefully the difference in growth properties between Ad2*ts*400 and its parent, burst size and the kinetics of virus replication were next determined. Confluent cell monolayers were infected with virus and allowed to undergo one infectious cycle. Virus yield was assayed by titration of the cell lysates on HeLa cells at 32.5°C (Table 1). Note that the growth of Ad2*ts*400 is indeed temperature sensitive in both HeLa and CV_1 cells. The yields of Ad2*ts*400 are less than 1 PFU per infected cell at 39.5°C, a reduction of 10^3 to 10^4-fold compared with growth at 32.5°C. Somewhat surprisingly, Ad2*hr*400 produced quite normal levels of infectious virus in CV_1 cells at 32.5°C (360 PFU per cell), even though it is cold sensitive for plaque formation. In comparison, Ad2*ts*400 produced nearly four times more virus (1,300 PFU per cell) under the same conditions in this experiment. Ad2*hr*400 and Ad2*ts*400 showed similar yields in HeLa cells at 32.5°C (440 and 240 PFU per cell, respectively).

The kinetics of growth of these two viruses at 32.5°C was next compared. At the completion of one infectious cycle, Ad2*ts*400-infected CV_1 cells yielded three to eightfold more infectious virus than did Ad2*hr*400-infected CV_1 cells (Fig. 2a). Both viruses, however, grew with similar kinetics. In contrast, Ad2*ts*400 and Ad2*hr*400 grew with similar kinetics and yield in HeLa cells at 32.5°C (Fig. 2b). This experiment was repeated twice more, and very similar results were obtained (data not shown). These results are also consistent with the virus yields shown in Table 1. We conclude that Ad2*ts*400 contains a mutation that allows the virus to produce higher yields in CV_1 cells at 32.5°C, but has little if any effect on the kinetics of viral growth. This mutation has no apparent effect on growth in HeLa cells at 32.5°C. Since several rounds of viral growth are required to produce a visible plaque, the three- to eightfold difference in virus yield is probably responsible for the different plaquing characteristics of Ad2*ts*400 and Ad2*hr*400 on CV_1 cells at 32.5°C.

As can be seen in Table 1 and Fig. 2a, Ad2*hr*400 produces quite normal levels of progeny virus in CV_1 cells at 32.5°C. Therefore, it may not be accurate to characterize its growth in CV_1 cells as cold sensitive. Plaque formation by Ad2*hr*400 on CV_1 cells, however, is cold sensitive relative to Ad2*ts*400. Thus, for the sake of consistency and clarity, we will continue to refer to the growth of Ad2*hr*400 and Ad2*ts*400 in CV_1 cells as cold sensitive and cold resistant, respectively.

Late protein synthesis in Ad2*ts*400-infected cells. Based on our selection rationale, we anticipated that the cold-resistant temperature-sensitive mutation(s) of Ad2*ts*400 resided in the DBP gene. Because a DBP mutation might directly (i.e., host range) or indirectly (i.e., DNA replication negative) affect late gene expression, we compared the patterns of late protein synthesis in Ad2-, Ad2*hr*400-, and Ad2*ts*400-infected cells. HeLa or CV_1 monolayers were infected with virus and incubated at 32.5 or 39.5°C until late times after infection. The cells were then pulse-labeled with [^{35}S]methionine, and

TABLE 1. Yields of virus in HeLa and CV_1 cells at 32.5 and 39.5°C

Virus[a]	Cell line	PFU/cell[b]	
		32.5°C	39.5°C
Ad2	HeLa	770	430
Ad2*hr*400	HeLa	440	310
Ad2*ts*400	HeLa	240	0.17
Ad2	CV_1	10	20
Ad2*hr*400	CV_1	360	400
Ad2*ts*400	CV_1	1,300	0.03

[a] Confluent monolayers were infected at a multiplicity of 20 PFU per cell.

[b] Progeny virus were harvested after 4 and 2 days of incubation at 32.5 and 39.5°C, respectively, and then titrated on HeLa cells at 32.5°C.

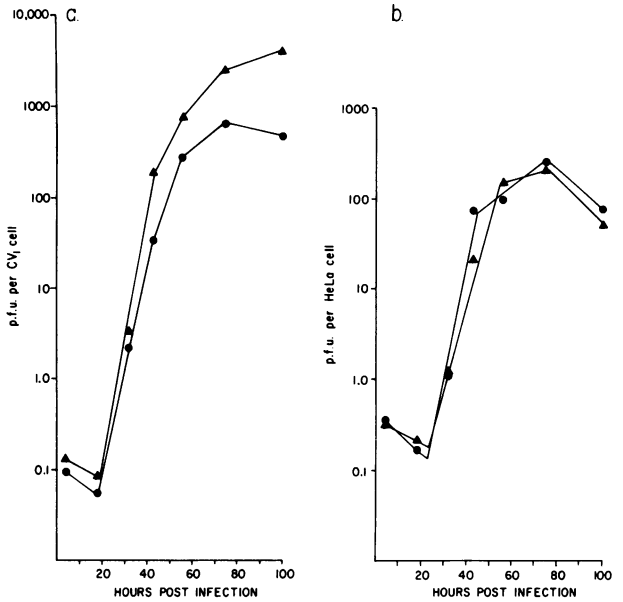

FIG. 2. Growth of Ad2*hr*400 (●) and Ad2ts400 (▲) at 32.5°C. Confluent monolayers of CV_1 (a) or HeLa (b) cells were infected with virus at 20 PFU per cell. After a 70-min adsorption at 37°C, the infected cells were incubated at 32.5°C. At various times after infection, cultures were frozen at −20°C. Virus yield was determined by titration of the cell lysates on HeLa monolayers at 32.5°C and is expressed as PFU per cell.

the total cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis. All three viruses showed similar patterns of late viral protein synthesis in HeLa cells at 32.5°C (Fig. 3a). At 39.5°C, in contrast, whereas Ad2 and Ad2*hr*400 both showed a normal pattern of protein synthesis in HeLa cells, Ad2*ts*400 failed to synthesize any late viral proteins. CV_1 cells infected with Ad2, both at 32.5 and 39.5°C, showed the typical abortive pattern of protein synthesis (Fig. 3b), i.e., some late adenovirus proteins were made in nearly normal amounts (100K protein), whereas the synthesis of others was reduced moderately (protein II) or severely (fiber protein) (31, 41). Ad2*hr*400- and Ad2*ts*400-infected CV_1 cells incubated at 32.5°C and Ad2*hr*400-infected CV_1 cells incubated at 39.5°C all showed normal synthesis of the late viral proteins, including fiber. CV_1 cells infected with Ad2*ts*400 at 39.5°C, however, failed to synthesize any late viral proteins. The temperature-sensitive mutation of *ts*400 thus appears to restrict the virus from entering the late phase of infection at 39.5°C in either human or monkey cells.

In contrast to the situation at 39.5°C, at 32.5°C Ad2*ts*400-infected CV_1 cells incorporated more radioactivity into late viral proteins during the pulse-label than did Ad2*hr*400-infected CV_1 cells (Fig. 3b). This result has been observed in several other experiments and is consistent with the observation that Ad2*ts*400 produces a larger burst size than does Ad2*hr*400 in CV_1 cells at 32.5°C. To pursue this observation in more depth we employed another established line of African green monkey kidney cells, called CV_c, in which adenovirus replication is even more restricted. At 32.5°C, virtually no late viral proteins were synthesized in Ad2-infected CV_c cells, irrespective of multiplicity of infection (Fig. 4). Coinfection with Ad2 plus SV40, as expected, allowed normal late gene expression. Both Ad2*hr*400- and Ad2*ts*400-infected CV_c cells produced late viral proteins at 32.5°C, but, as in CV_1 cells, the rate of late viral protein synthesis appeared greater in Ad2*ts*400-infected cells. This difference was most striking for the fiber protein. Cells infected with Ad2*ts*400 appeared to synthesize at least 10-fold more fiber during the pulse-label than did Ad2*hr*400-infected cells, although both viruses made nearly equivalent amounts of the 100K protein. These results suggest that the cold sensitivity of Ad2*hr*400 in monkey cells is due to a specific defect in late gene expression that is related to that observed in WT Ad2 infection of monkey cells, where again the synthesis of the fiber protein is most dramatically affected.

Ad2*ts*400 is temperature sensitive for DNA replication. The observation that no late viral proteins were synthesized in Ad2*ts*400-infected cells at 39.5°C suggested that the virus was temperature sensitive for DNA replication since the viral genome must be replicated before it can serve as a template for late gene expression (64). To verify this supposition, viral DNA replication was analyzed. Duplicate plates of either HeLa or CV_1 cells were infected with Ad2*hr*400, Ad2*ts*400, or the known DNA-negative mutant Ad5*ts*125 and incubated at 32.5°C until well into the DNA replication phase (29 h p.i.). At this point, one plate from each duplicate infection was shifted to 39.5°C, and after 4 h, all plates were labeled with [^{3}H]thymidine for 2.5 h. The cells were then harvested, and low-molecular-weight DNA was prepared by a modified Hirt extraction procedure (35). Each DNA sample was digested with the restriction enzyme *Hin*dIII and electrophoresed on a 1.2% agarose gel. Ethidium bromide staining of the gel showed that roughly equivalent amounts of viral DNA were present in each preparation (Fig. 5a). Because a large amount of viral DNA had been synthesized

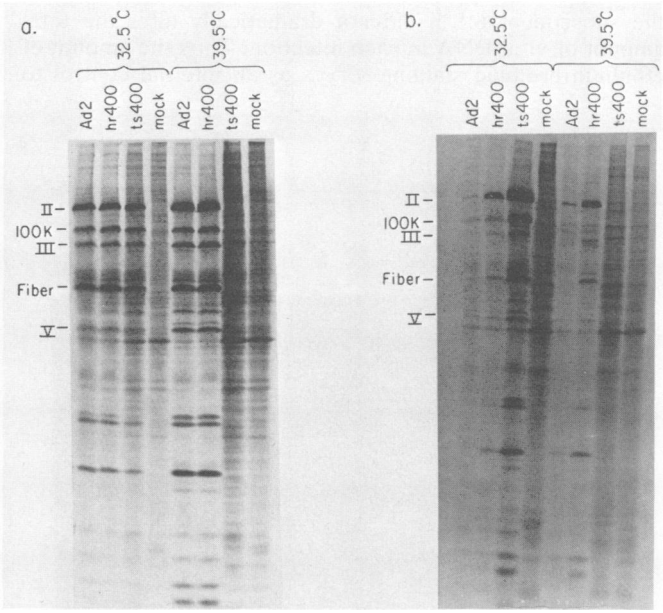

FIG. 3. In vivo synthesis of viral proteins in HeLa and CV_1 cells at 32.5 and 39.5°C. Monolayer cultures of HeLa (a) or CV_1 (b) cells were infected with virus and incubated at the temperature indicated. At late times after infection (24 and 47 h for HeLa cells at 39.5 and 32.5°C, respectively; 29 and 67 h for CV_1 cells at 39.5 and 32.5°C, respectively), the monolayers were labeled with 40 μCi of [^{35}S]methionine per ml for 1 h at 39.5°C and 2 h at 32.5°C. An equal fraction of each labeled cell lysate was electrophoresed on a 15% SDS-polyacrylamide gel. The labeled proteins were visualized by autoradiography of the dried gel. The positions of several late adenovirus proteins are indicated.

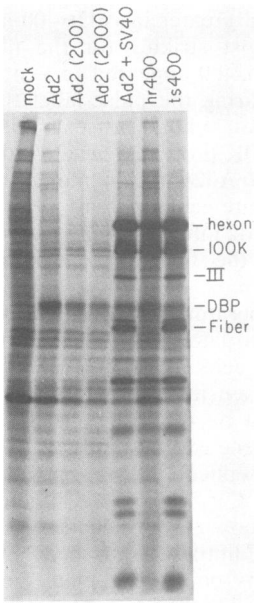

FIG. 4. In vivo synthesis of viral proteins in CV_c cells at 32.5°C. Cell monolayers were labeled, and protein synthesis was analyzed as in Fig. 3, except that labeling was for 2 h at 48 h p.i. Infections were done at a multiplicity of 20 PFU per cell, except in two Ad2 infections, where the multiplicity of infection was raised to 200 and 2,000 PFU per cell (indicated by numbers in parentheses). In the Ad2-SV40 coinfection, SV40 was used at a multiplicity of infection of 100 PFU per cell.

before the shift-up, the additional DNA synthesized during the subsequent 6.5 h did not dramatically alter the total amount of viral DNA in each infection. Thus the amount of ethidium bromide staining serves as an internal control to show that viral DNA was extracted with approximately equal efficiency (factor of 2) from each infection.

Analysis of the fluorograph of the same gel (Fig. 5b) indicated that little [^{3}H]thymidine was incorporated into Ad5*ts*125 DNA after shift-up to 39.5°C in both HeLa and CV_1 cells, as expected. Similar results were observed in Ad2*ts*400-infected HeLa or CV_1 cells after shift-up. In contrast, Ad2*hr*400 incorporated more rather than less [^{3}H]thymidine into viral DNA after shift-up to 39.5°C, probably due to a generalized increase in the rate of metabolism at the higher temperature. Thus Ad2*ts*400, like Ad5*ts*125, is temperature sensitive for viral DNA replication.

Ad2*ts*400 is in the same temperature-sensitive complementation group as Ad5*ts*125 and Ad5*ts*107. The phenotype of Ad2*ts*400 and the rationale for its selection suggested that the temperature-sensitive lesion probably resided in the DBP gene. Complementation analysis with the known DBP mutants Ad5*ts*125 and Ad5*ts*107, and Ad5*dl*434, an Ad5 deletion mutant (coordinates 2.6 to 8.7) lacking parts of early regions 1A and 1B (D. Solnick, unpublished data), confirmed this suspicion. HeLa cells were infected either singly or in pairwise combinations with these mutants and incubated at 39.5°C for 3 days. The cell lysates were titrated on 293 cells at 32.5°C to determine virus yield (Table 2). As expected, coinfections with Ad5*dl*434 plus Ad5*ts*125 or Ad5*ts*107 yielded large numbers of progeny virus, whereas coinfection with Ad5*ts*125 plus Ad5*ts*107 did not. Mixed infection with Ad5*dl*434 plus Ad2*ts*400 yielded large numbers of progeny, but the Ad2*ts*400 plus Ad5*ts*125 or Ad5*ts*107 coinfections did not. Ad2*ts*400, Ad5*ts*125, and Ad5*ts*107 are therefore members of the same temperature-sensitive complementation group.

Physical mapping of the Ad2*ts*400 temperature sensitivity mutation. The temperature-sensitive mutation of Ad2*ts*400 was physically localized on the adenovirus genome by marker rescue analysis (26, 43, 63). 293 cells were cotrans-

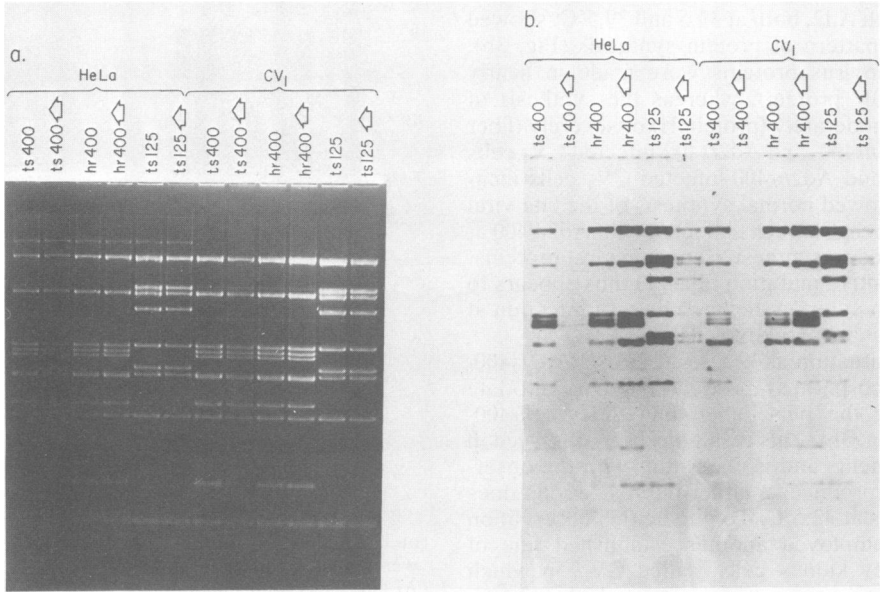

FIG. 5. Analysis of viral DNA replication after temperature shift-up. Duplicate plates of confluent HeLa or CV_1 monolayers were infected with Ad2*hr*400, Ad2*ts*400, or Ad5*ts*125 and incubated at 32.5°C until 29 h p.i. At this time, one plate (indicated by arrow in figure) from each duplicate infection was shifted to 39.5°C. After 4 more h, all plates were labeled with 50 μCi of [^{3}H]thymidine per ml for 2.5 h. Low-molecular-weight DNA was extracted from each infection, and an equal fraction of each DNA preparation was digested with *Hin*dIII and electrophoresed on a 1.2% agarose gel. (a) Ethidium bromide stain of the gel; (b) Autoradiogram of the gel after treatment for fluorography. The major DNA bands correspond to expected adenovirus *Hin*dIII restriction fragments for serotypes 2 (Ad2*ts*400 and Ad2*hr*400) and 5 (Ad5*ts*125).

TABLE 2. Complementation between adenovirus mutants in HeLa cells at 39.5°C

Virus[a]	PFU/cell[b]
Ad2*hr*400	200
Ad5*ts*125	0.03
Ad5*ts*107	0.38
Ad5*dl*434	0.16
Ad2*ts*400	0.13
Ad5*ts*125 + Ad5*ts*107	0.10
Ad5*ts*125 + Ad5*dl*434	420
Ad5*ts*107 + Ad5*dl*434	23
Ad2*ts*400 + Ad5*ts*125	0.07
Ad2*ts*400 + Ad5*ts*107	0.10
Ad2*ts*400 + Ad5*dl*434	250

[a] Confluent HeLa monolayers were infected at multiplicity of 20 for single virus infections or 10 each for the coinfections. The infected cells were incubated for 68 h at 39.5°C before being harvested.

[b] The cell lysates were titrated on 293 cells at 32.5°C; 293 cells contain and express adenovirus early regions 1A and 1B and thus support the growth of Ad5*dl*434.

fected with full-length Ad2*ts*400 DNA-terminal protein complex plus purified DNA restriction fragments derived from nontemperature-sensitive virus (Ad2, Ad2*hr*400, Ad2*hr*403). The transfections were allowed to proceed through one infectious cycle at 32.5°C, and the cell lysates were then passaged once or twice on HeLa cells at 39.5°C to enrich for nontemperature-sensitive recombinants. The final yield of nontemperature-sensitive virus was determined by titration on HeLa cells at 39.5°C (Table 3). *Eco*RI fragments spanning the entire genome were tested for their ability to rescue. Only the *Eco*RI B fragment (coordinates 58.5 to 70.7) efficiently rescued the temperature-sensitive lesion. *Bgl*II and *Kpn*I fragments were used to further localize the temperature-sensitive mutation within this area of the genome. Both the *Bgl*II J fragment (coordinates 60.2 to 63.6) and the *Kpn*I E fragment (coordinates 61.3 to 71.4) were able to rescue. The temperature-sensitive mutation of Ad2*ts*400 therefore maps between coordinates 61.3 and 63.6 of the Ad2 genome, placing it in the C-terminal half of the DBP gene at a position analogous to that of the Ad5 DBP mutants Ad5*ts*125 and Ad5*ts*107 (44–46).

Ad2*ts*400 is a double mutant. Ad2*ts*400 has two phenotypes that distinguish it from its progenitor, Ad2*hr*400. First, it is fully cold resistant for late gene expression and growth in monkey cells. Second, Ad2*ts*400 is temperature sensitive for DNA replication and growth in both monkey and human cells. The rationale behind our selection scheme was to create both cold-resistant and temperature-sensitive phenotypes in one mutational step. If such were the case, we would expect that the nontemperature-sensitive recombinants derived in the marker rescue experiments would concommitantly have lost their cold-resistant phenotype.

To test this, virus from plaques resulting from the various crosses described above were isolated and titrated on HeLa and CV_1 monolayers at both 39.5 and 32.5°C. As expected, none of the rescued virus exhibited the temperature-sensitive phenotype. All of the recombinants were also able to grow efficiently on monkey cells. This was also expected, since the *Eco*RI B and *Kpn*I E fragments were derived from host range virus, whereas the *Bgl*II J fragment, derived from WT Ad2, does not include the site of the host range mutation. Surprisingly, however, the rescued virus stocks fell into two classes with respect to their plaquing characteristics on CV_1 cells at 32.5°C. Both viruses that had been rescued with the Ad2 *Bgl*II J fragment retained the ability to form large, rapidly appearing plaques. In contrast, both viruses rescued with the Ad2*hr*403 *Kpn*I E fragment reverted to the small-plaquing phenotype characteristic of Ad2*hr*400 (Ad2*hr*403, like Ad2*hr*400, produces small plaques on CV_1 cells at 32.5°C). Viruses rescued with the *Eco*RI B fragment fell into both classes.

These results indicate that Ad2*ts*400 is a double mutant, because physical rescue of the temperature-sensitive mutation does not necessarily convert the cold-resistant phenotype to cold sensitivity. However, in some cases, purified restriction fragments were simultaneously able to convert both phenotypes. The *Eco*RI B fragment (coordinates 58.5 to 70.7) and *Kpn*I E fragment (coordinates 61.3 to 71.4) had this ability and must presumably cover the cold-resistant mutation. The cold-resistant mutation thus lies between coordinates 61.3 and 70.7 on the adenovirus genome.

Physical mapping of the Ad2*ts*400 cold-resistant mutation. Initial attempts were made to physically map the Ad2*ts*400 cold-resistant mutation by a modified marker rescue technique. Genomic DNA from Ad2*hr*400 plus individual restriction fragments derived from Ad2*ts*400 were cotransfected into 293 cells. After completion of one infectious cycle at 32.5°C, the cell lysates were passaged on either CV_1 or CV_c cells at 32.5°C to enrich for cold-resistant recombinants. The results of these experiments were equivocal due to high background levels of unrescued Ad2*hr*400 virus. Presumably the selection for cold resistance in monkey cells was not strong enough to give unambiguous results.

To increase the selection pressure the above experiment was repeated with WT Ad2 as the genomic background rather than Ad2*hr*400. There existed two problems to this approach. First, we needed to ensure that we were mapping the Ad2*ts*400 cold-resistant mutation and not simply remap-

TABLE 3. Physical mapping of the Ad2*ts*400 temperature-sensitive mutation by marker rescue

Expt	Restriction fragment(s)	Coordinates	PFU/ml[a]
1	None		<10
	Ad2*hr*400 *Eco*RI-A	0–58.5	8,500
	Ad2*hr*400 *Eco*RI-B	58.5–70.7	120,000
	Ad2*hr*400 *Eco*RI-C	89.7–100	200
	Ad2*hr*400 *Eco*RI-D	75.9–84.0	500
	Ad2*hr*400 *Eco*RI-E	84.0–89.7	<10
	Ad2*hr*400 *Eco*RI-F	70.7–75.9	<10
	Ad2 *Bgl*II-C + *Bgl*II-D[b]	63.6–77.9, 45.3–60.2	300
	Ad2 *Bgl*II-J	60.2–63.6	25,000
2	None		<10
	Ad2*hr*403 *Eco*RI-A	0–58.5	<10
	Ad2*hr*403 *Eco*RI-B	58.5–70.7	3,100
	Ad2*hr*403 *Kpn*I-D	47.4–61.3	400
	Ad2*hr*403 *Kpn*I-E	61.3–71.4	55,000
3	None		<10
	Ad2 *Bgl*II-C + *Bgl*II-D[b]	63.6–77.9, 45.3–60.2	<10
	Ad2 *Bgl*II-J	60.2–63.6	3,800

[a] Intact Ad2*ts*400 DNA-terminal protein complex was mixed with the restriction fragments indicated and transfected into 293 cells. The progeny resulting from such crosses were passaged once (experiments 1 and 3) or twice (experiment 2) in HeLa cells at 39.5°C, and the resulting progeny were titrated on HeLa cell monolayers at 39.5°C. The numbers given are the averages of two independent crosses.

[b] The *Bgl*II fragments C and D cannot be separated by electrophoresis on agarose gels.

ping the original host range mutation. Growth in monkey cells at 32.5°C should select for the cold-resistant phenotype of Ad2*ts*400, although as just noted this selection is not strong. In addition, rescued virus that are truly cold resistant should form large, rapidly appearing plaques on CV_1 cells and thus should be distinguishable from virus that have acquired only the Ad2*hr*400 mutation. The second problem concerns whether the cold-resistant mutation would manifest itself in a wild-type genetic background. The cold-resistant phenotype might require both the original Ad2*hr*400 mutation plus the cold-resistant mutation. A restriction fragment that carries only the latter mutation may not be able to bestow a cold-resistant or even a host range phenotype. However, from the earlier analysis of non-temperature-sensitive rescued virus, we knew that the cold-resistant mutation mapped relatively close to the Ad2*hr*400 mutation. Thus we suspected that both mutations would often be present on the same Ad2*ts*400 restriction fragment.

WT Ad2 DNA-terminal protein complex plus Ad2*ts*400 restriction fragments were cotransfected into 293 cells at 32.5°C. The lysates of the transfected cells were passaged one time on CV_c cells at 32.5°C to enrich for cold-resistant recombinants, and final virus yield was determined by titration on CV_1 cells at 32.5°C (Table 4). Plaques were counted after 18 days. Since Ad2*hr*400 plaques do not appear until approximately day 21, all virus plaquing at this point presumably carry the cold-resistant mutation. As expected, Ad2*ts*400 but not Ad2*hr*400, unfractionated *Xho*I fragments were able to confer the cold-resistant phenotype on WT Ad2. Among the crosses performed with purified *Xho*I fragments, only *Xho*I-E (coordinates 53 to 66) crosses were able to yield cold-resistant virus. *Bgl*II and *Kpn*I fragments were used to further localize the cold-resistant mutation on the adenovirus genome. Both *Bgl*II-C–*Bgl*II-D and *Kpn*I E fragments were able to efficiently confer the cold-resistant phenotype. This places the cold-resistant mutation between coordinates 63.6 and 66.

The cold-resistant mutation thus maps in the N-terminal segment of the DBP gene, as does the original Ad2*hr*400 mutation (44). We are confident that the marker rescue experiments map the cold-resistant mutation and not simply remap the original Ad2*hr*400 mutation for two reasons. First, crosses performed with unfractionated *Xho*I restriction fragments derived from Ad2*hr*400 yielded no progeny virus. Second, progeny virus obtained in successful crosses yielded large, rapidly forming plaques that were visible by day 18. Since both the Ad2*hr*400 and cold-resistant mutations map to the same restriction fragments, we do not know whether the cold-resistant mutation alone can confer the cold-resistant phenotype or whether both mutations are necessary.

Cold-resistant recombinants obtained in the crosses were examined to see whether they had simultaneously acquired the temperature-sensitive phenotype. The results were consistent with the earlier mapping data, which localized the temperature-sensitive mutation to coordinates 61.3 to 63.6. Two of two recombinants obtained in the *Xho*I-E (coordinates 53 to 66) cross acquired the temperature-sensitive phenotype, whereas one of two recombinants obtained in the *Kpn*I-E (coordinates 61.3 to 71.4) cross was temperature sensitive. As expected, neither of the two recombinants derived in the *Bgl*II-C–*Bgl*II-D cross (coordinates 63.6 to 77.9 and 45.3 to 60.2) had acquired the temperature-sensitive phenotype.

Early mRNA accumulation in Ad2*ts*400-infected cells at the nonpermissive temperature. Experiments were performed to determine whether Ad2*ts*400, like Ad5*ts*125, accumulated early viral mRNA at the nonpermissive temperature (7, 10, 11, 50). Suspension cultures of HeLa cells were infected at high multiplicity with Ad2 or Ad2*ts*400 and incubated at 41°C in the presence of araC to inhibit DNA replication. At 6, 12, and 18 h p.i., samples of cells were taken, and cytoplasmic RNA was extracted. Polyadenylated RNA selected from 100 μg of each RNA preparation was electrophoresed on a denaturing agarose gel, transferred to diazobenzyloxymethyl paper, and probed with radioactively labeled, cloned DNAs specific for each early region. Differences in early mRNA accumulation between Ad2 and Ad2*ts*400 were not readily apparent at 6 h p.i. (Fig. 6). This was confirmed by densitometric tracings of the autoradiograms, which showed that at 6 h p.i. the steady-state levels of early mRNAs produced by the two viruses were within a factor of two. By 12 and 18 h, however, Ad2*ts*400 had accumulated higher steady-state levels of early viral mRNAs than had Ad2. At 18 h p.i., the amount of accumulation relative to the WT infection as assessed by densitometry was greatest for early region 1B mRNAs (eightfold), whereas the other early regions (1A, 2A,

TABLE 4. Physical mapping of the Ad2*ts*400 cold-resistant mutation by marker rescue

Expt	Restriction fragment(s)	Coordinates	PFU/ml[a]
1	None		<10
	Unfractionated Ad2*hr*400 *Xho*I[b]		<10
	Unfractionated Ad2*ts*400 *Xho*I[b]		830
	Ad2*ts*400 *Kpn*I-D	47.4–61.3	<10
	Ad2*ts*400 *Kpn*I-E	61.3–71.4	5,000
	Ad2*ts*400 *Bgl*II-C + *Bgl*II-D[c]	45.3–60.2, 63.6–77.9	3,100
	Ad2*ts*400 *Bgl*II-J	60.2–63.6	<10
2	None		<10
	Ad2*ts*400 *Xho*I-A	26.5–53	<10
	Ad2*ts*400 *Xho*I-B + *Xho*I-C + *Xho*I-D[c]	83–100, 66–83, 0–15.5	<10
	Ad2*ts*400 *Xho*I-E	53–66	930
	Ad2*ts*400 *Xho*I-F	15.5–22	<10
	Ad2*ts*400 *Xho*I-G	22–26.5	<10

[a] Intact Ad2 DNA-terminal protein complex was mixed with the restriction fragments indicated and transfected into 293 cells. The progeny resulting from such crosses were passaged once in CV_c cells at 32.5°C, and titers of the resulting progeny were determined on CV_1 monolayers at 32.5°C. The numbers given are the averages of two independent crosses.

[b] Neither Ad2*hr*400 or Ad2*ts*400 unfractionated *Xho*I fragments were infectious in the absence of Ad2 DNA-terminal protein complex.

[c] The *Bgl*II-C and *Bgl*II-D and the *Xho*I-B, *Xho*I-C, and *Xho*I-D restriction fragments cannot be separated by electrophoresis on agarose gels.

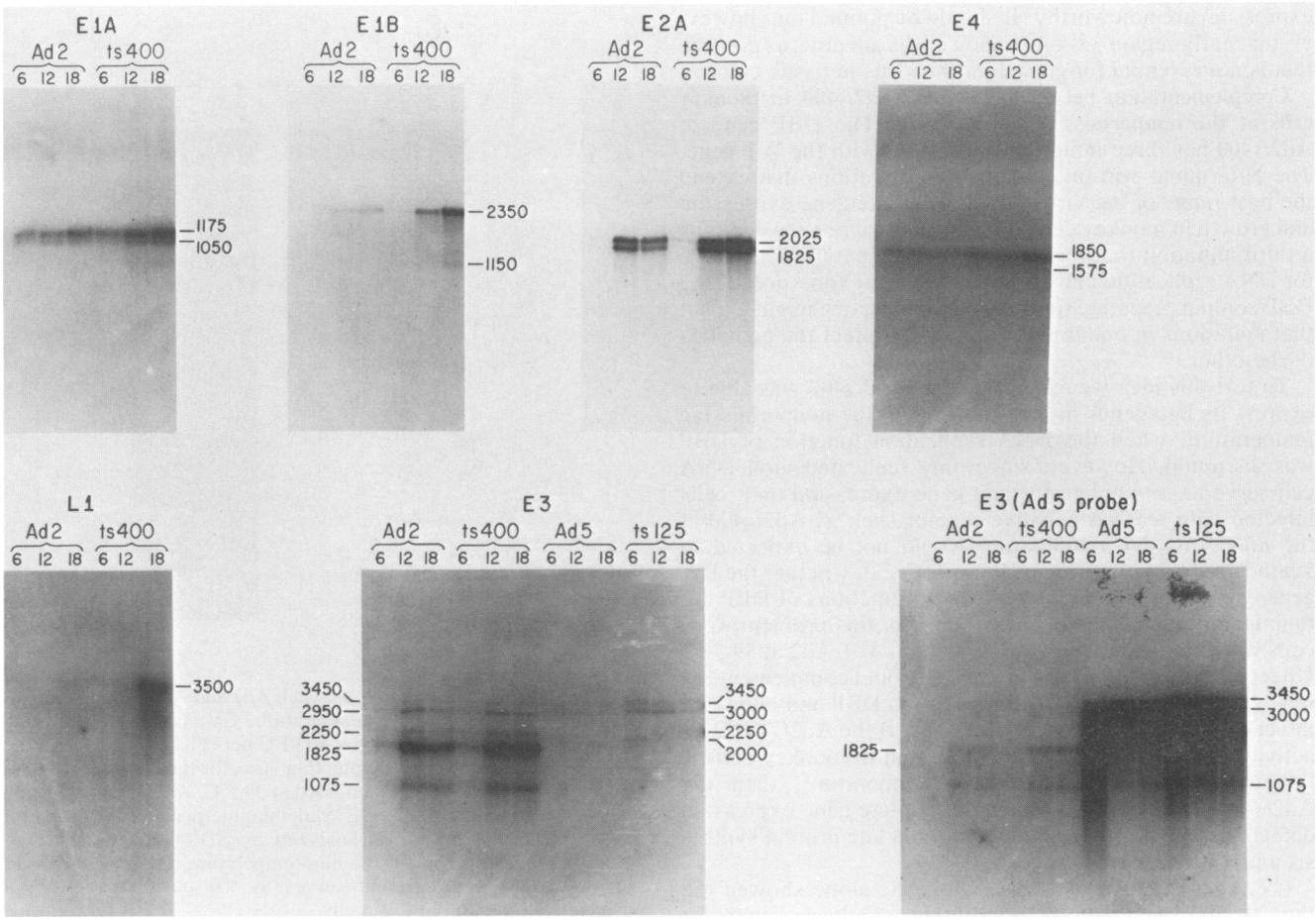

FIG. 6. Early viral mRNA accumulation in HeLa cells at 39.5°C. Suspension cultures of HeLa cells were infected with Ad2, Ad2*ts*400, Ad5, or Ad5*ts*125 and incubated at 41°C in the presence of araC to inhibit DNA replication. Cytoplasmic RNA was extracted from approximately equal numbers of cells at 6, 12, and 18 h p.i. Samples (100 μg) of each RNA preparation were fractionated on oligodeoxythymidylic acid-cellulose columns, and the polyadenylated fractions were electrophoresed through a denaturing 1.2% agarose gel containing 10 mM methyl mercury hydroxide. The RNA was blotted onto diazobenzyloxymethyl paper and probed with nick-translated DNAs specific for each adenovirus early region (indicated above each panel). The probes used were plasmid clones of Ad2 DNA, except the one early region 3 blot indicated, which was probed with a purified Ad5 restriction fragment specific for E3. The numbers above each track indicate the hours after infection that the RNA was isolated, and the brackets indicate the infecting virus. The numbers beside each panel indicate the approximate size in nucleotides of the mRNA species.

3, and 4) were overproduced to lesser extents (two- to threefold). The mRNA species made in both infections were in general similar and similar in size and relative abundance to the species previously reported in Ad2-infected cells treated with araC (4, 9, 16).

The late L1 mRNA family, which has previously been reported to be expressed during the early phase of infection (1, 16, 51, 62), was also analyzed (Fig. 6). A single 3,500-nucleotide mRNA was present at 18 h in Ad2*ts*400-infected cells, but was not detectable in the WT infection. Other groups have reported the presence of a single L1 cytoplasmic RNA during the early phase, although its size has been reported as 4,000 (51) and 2,900 (1) nucleotides.

Early mRNA accumulation was also examined in Ad5- and Ad5*ts*125-infected cells. In general, the overproduction of early messages relative to the WT in Ad5*ts*125-infected cells was similar to that observed in Ad2*ts*400-infected cells, although the kinetics of early mRNA accumulation appeared to differ between the two viral serotypes (data not shown). A striking serotypic difference was noted, however, in the early region 3 mRNA species hybridizing to the Ad2-specific probe (Fig. 6). To see whether this result was due to sequence heterology or serotypic differences in the pattern of early region 3 mRNA expression (or to both), an Ad5-specific probe covering the same viral coordinates was hybridized to a similar Northern blot of the same RNA preparation. Analysis of this blot (Fig. 6) showed that both the above explanations were correct. This region of the adenovirus genome shows large serotypic sequence heterology, since the patterns of hybridization by the two probes were quite dissimilar. In addition, early region 3 shows serotypic differences in the pattern of mRNA expression. In particular, the major 1,825-nucleotide Ad2 species was not detected in either the Ad5 or Ad5*ts*125 infections. Evidence for extensive sequence differences between serotypes 2 and 5 in this region of the adenovirus genome has been reported previously (27). Furthermore, Flint et al. (22), using saturation hybridization techniques, concluded that Ad5 early region 3 mRNA corresponded in position to Ad2 mRNA, but also contained some additional adjacent sequences. Since the closely related Ad2 and Ad5 are often used interchangeably, these differences in nucleotide sequence and mRNA

expression are noteworthy. It should be pointed out, however, that early region 3 is one region of the adenovirus genome that is nonessential for growth of the virus in tissue culture.

Complementation between Ad2 and Ad2*ts*400 in monkey cells at the nonpermissive temperature. The DBP gene of Ad2*ts*400 has three mutations compared with the WT gene. The N-terminal portion contains two mutations that extend the host range of the virus by allowing late gene expression and growth in monkey cells. The C-terminal portion contains a third mutation that makes the virus temperature sensitive for DNA replication and proper early gene expression. If the DBP contains separate functional domains, one might expect that mutations in one domain would not affect the activities of the other.

To test this idea we asked whether Ad2*ts*400 was able to express its late genes in monkey cells at the nonpermissive temperature, when the DNA replication function of DBP was disrupted. However, since only replicated viral DNA can serve as a template for late gene expression (64), cells infected with a DNA-negative mutant such as Ad2*ts*400 at the nonpermissive temperature would not be expected to synthesize late viral proteins irrespective of whether the late gene expression and DNA replication functions of DBP are functionally independent. To circumvent this problem, CV_1 cells were coinfected with Ad2*ts*400 plus WT Ad2 at 39.5°C. Under these conditions the WT DBP should complement the DNA replication defect of the Ad2*ts*400 DBP and allow the infection to proceed into the late phase. If the Ad2*ts*400 DBP activity that allows late gene expression in monkey cells is functional at the nonpermissive temperature, then the Ad2*ts*400 DBP should complement the late gene expression defect of the WT DBP and allow normal late protein synthesis and viral growth.

CV_1 cells infected at 39.5°C with Ad2 alone showed the typical abortive pattern of protein synthesis, whereas Ad2*ts*400-infected cells, as expected, are restricted to the early phase of infection (Fig. 7a). Cells coinfected with Ad2 plus Ad2*ts*400, on the other hand, showed a productive pattern of late protein synthesis, with all late proteins, including fiber, being made in normal amounts. Virus yield was also greatly enhanced in the mixed infections (Table 5). Low levels of progeny virus were produced in cells infected with Ad2 or Ad2*ts*400 alone (9.0 and 1.3 PFU per cell, respectively), but coinfection yielded normal levels of infectious virus (450 PFU per cell). The yields of both Ad2 and Ad2*ts*400 were enhanced to similar extents in the mixed infection.

The results of this experiment suggest that the ability of Ad2*ts*400 to express its late genes in monkey cells is not temperature sensitive. A repeat of this experiment in CV_c cells, where the block to adenovirus growth is more stringent, showed that although complementation between Ad2 and Ad2*ts*400 occurred, it was not as complete as in CV_1 cells (Fig. 7b; see below).

Ad2*ts*400 late gene expression in monkey cells after temperature shift-up. Although the previous set of results argues that the late gene expression activity of DBP functions independently of its DNA replication activity, the possibility exists that these activities are not independent and that the observed complementation was simply due to the WT DBP protecting the mutant DBP against heat inactivation, possibly as part of a multimeric complex. To rule out this possibility temperature shift experiments were performed with Ad2*ts*400 in the absence of complementing virus.

CV_c cells were infected with Ad2, Ad2*ts*400, or a non-temperature-sensitive derivative of Ad2*ts*400 called

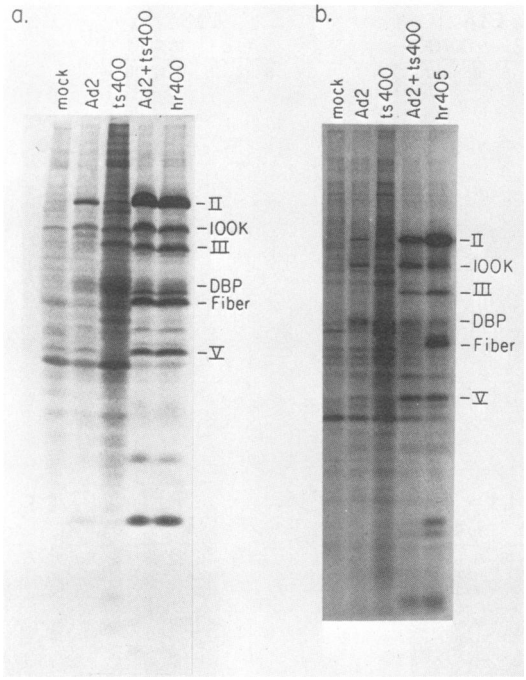

FIG. 7. Complementation between Ad2 and Ad2*ts*400 in monkey cells at the nonpermissive temperature. CV_1 (a) or CV_c (b) cells were infected at a multiplicity of 40 PFU per cell for single infections and 20 PFU per cell of each infecting virus for the coinfections. The infected monolayers were incubated at 39.5°C. At 30 h p.i., the cells were labeled with 40 μCi of [^{35}S]methionine per ml for 1 h, and in vivo protein synthesis was analyzed by SDS-polyacrylamide gel electrophoresis. Ad2*hr*405 is a non-temperature-sensitive derivative of Ad2*ts*400 (see text) and serves as the positive control for productive infection in CV_c cells.

Ad2*hr*405. Ad2*hr*405 was derived during the marker rescue experiments from a cross between the intact Ad2*ts*400 genome and a purified *Bgl*II J restriction fragment (coordinates 60.2 to 63.6) of WT Ad2. Thus, Ad2*hr*405 is presumably genetically identical to Ad2*ts*400, except for lacking the temperature-sensitive mutation between coordinates 61.3 and 63.6. The infections were maintained at 32.5°C for 29 h to allow them to proceed into the beginning of the late phase. At this time, one of a series of replicate infections was pulse-labeled with either [^{3}H]thymidine or [^{35}S]methionine, and the remaining infections were transferred to 39.5°C. As a control, the DNA synthesis inhibitor araC was added to one set of Ad2*hr*405-infected cells incubated at 39.5°C. At 4, 8, and 12 h after the temperature shift-up, plates from each infection (Ad2, Ad2*hr*405, Ad2*hr*405 plus araC, and Ad2*ts*400) were pulse-labeled with either [^{3}H]thymidine or [^{35}S]methionine. One set of plates was pulse-labeled with [^{35}S]methionine immediately after incubation at 39.5°C.

Low-molecular-weight DNA was extracted from cells labeled with [^{3}H]thymidine, and an equal sample from each preparation was hybridized to Ad2 DNA immobilized on nitrocellulose filters. The rate of DNA synthesis in either Ad2- or Ad2*hr*405-infected CV_c cells increased during the 12 h after the temperature shift-up (Fig. 8a). When araC was added to the Ad2*hr*405 infections at the time of the shift-up, however, DNA synthesis was markedly reduced. Ad2*ts*400 DNA synthesis, which at the time of the shift-up was occuring at a slightly faster rate than that of Ad2*hr*405, was practically undetectable at 4, 8, and 12 h after the shift-up.

TABLE 5. Complementation between Ad2 and Ad2*ts*400 in CV_1 cells at 39.5°C

Virus[a]	PFU/cell
Ad2	9.0[b]
Ad2*ts*400	1.3[c]
Ad2*hr*400	950[b]
Ad2 + Ad2*ts*400	170[b] + 280[c]

[a] CV_1 cells were infected at a multiplicity of 40, or 20 each for the Ad2 plus Ad2*ts*400 coinfection. The infected cells were incubated at 39.5°C for 48 h.
[b] Yield was determined by titration on HeLa cells at 39.5°C.
[c] Yield was determined by titration on CV_1 cells at 32.5°C.

The result is consistent with our earlier observation that Ad2*ts*400 is temperature sensitive for DNA replication.

The critical question is whether Ad2*ts*400 late gene expression is also temperature sensitive. Since ongoing DNA synthesis is not required for late gene expression (12), viral DNA replicated before the shift-up should continue to act as a template for late gene expression. We purposely chose to shift the temperature at a time early during the late phase, hoping to catch the infections at a point before steady-state levels of the late mRNAs had been reached. Thus, if Ad2*ts*400 late gene expression is not temperature sensitive, the rate of late viral protein synthesis should remain constant and perhaps even increase after the shift-up. If the late gene expression activity of the Ad2*ts*400 DBP is temperature sensitive, then, depending on the level at which the block to late protein synthesis occurs in monkey cells, viral protein synthesis may drop immediately (translational defect) or after some lag (transcriptional or RNA processing defect) after the shift-up. If viral mRNA synthesis is defective in monkey cells, then the length of this lag will depend on the stability of the preexisting viral mRNAs. Because fiber protein synthesis is most dramatically affected in monkey cells, alterations in its production are most easily followed after the shift-up. Since the half-life of fiber mRNA is approximately 2 h in CV_1 cells (K. P. Anderson and D. F. Klessig, unpublished data) and presumably also in CV_c cells, a significant drop in fiber protein synthesis should be apparent by 4 and certainly 8 h after the shift-up if no new functional fiber mRNA is being produced due to the inactivation of DBP's late gene expression function.

As expected, Ad2-infected CV_c cells showed no detectable fiber synthesis either before or 12 h after the temperature shift-up (Fig. 8b). In contrast, fiber expression was evident in Ad2*hr*405-infected cells and increased substantially during the shift-up period. When Ad2*hr*405-infected cells were treated with araC to block DNA replication, fiber synthesis increased between 0 and 4 h after the shift-up, appeared to level off, and then dropped somewhat at 12 h after the shift-up. A very similar pattern of fiber synthesis was seen in the Ad2*ts*400 infections. Although araC-treated Ad2*hr*405 infections showed slightly more fiber synthesis than did Ad2*ts*400 infections after the shift-up, this is probably the result of a greater number of DNA templates in these cells due to a less stringent block of DNA replication by araC

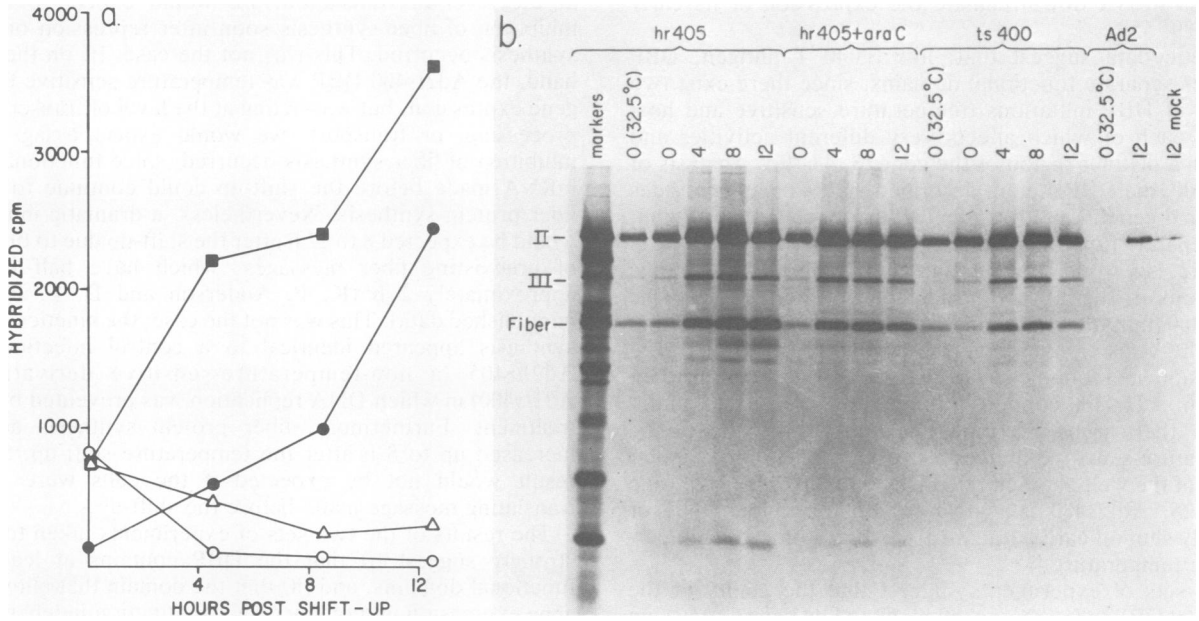

FIG. 8. DNA synthesis and late gene expression in Ad2*ts*400-infected monkey cells after temperature shift-up. Replicate cultures of CV_c monolayers were mock infected or infected with Ad2 (●), Ad2*hr*405 (■), or Ad*ts*400 (○) and incubated at 32.5°C for 29 h. At this time, infected monolayers from two plates of each infection were labeled at 32.5°C for 1 h with either 50 μCi of [^{3}H]thymidine per ml or 40 μCi of [^{35}S]methionine per ml. araC was added to one group of Ad2*hr*405-infected cells (△). These and all of the remaining infections were shifted to 39.5°C. At 0, 4, 8, and 12 h after shift-up, infected monolayers were labeled with either [^{3}H]thymidine or [^{35}S]methionine for 1 h at 39.5°C. (a) DNA synthesis after temperature shift-up. Low-molecular-weight DNA was prepared from [^{3}H]thymidine-labeled cells by a modified Hirt extraction. Equal fractions of each preparation were boiled in alkali and hybridized to Ad2 DNA immobilized on nitrocellulose filters. The zero time point represents cells labeled for 1 h at 32.5°C, and all other points represent cells labeled for 1 h at 39.5°C. (b) Fiber protein synthesis after temperature shift-up. Equal fractions of each [^{35}S]methionine-labeled protein extract were immunoprecipitated with polyclonal antibodies raised against native fiber protein, and the precipitated polypeptides were analyzed by electrophoresis on a 15% SDS–polyacrylamide gel. The numbers above each track denote the time after shift-up when the cells were labeled. The parentheses above some numbers indicate labeling was for 1 h at 32.5°C. Otherwise, labeling was at 39.5°C for 1 h. The position of the fiber and other late adenovirus proteins are indicated to the left of the panel.

than by the temperature-sensitive mutation (Fig. 8a). Since the actual amount of functional fiber mRNA or the efficiency of its translation actually increased after the shift-up, the late gene expression function of DBP must remain active even though its DNA replication function is disrupted.

DISCUSSION

Few multifunctional eucaryotic proteins have been studied in great detail, in part due to the great difficulty, at least until recently, of obtaining large numbers of mutants for genetic and biochemical analyses. One exception is the SV40 large T antigen. A variety of genetic and biochemical studies indicate that T antigen is a multifunctional protein containing separate functional domains (for review, see reference 32). A large body of evidence suggests that the adenovirus-specified, 72-kd DBP is another eucaryotic protein that is multifunctional. The similarities between DBP and T antigen are striking. Both are phosphorylated, early viral gene products that are necessary for viral DNA replication and the repression of early gene expression during the late phase of infection. Both are DBPs; T antigen binds specifically to the SV40 origin of replication, whereas DBP binds non-specifically to single-stranded DNA. In addition, the two proteins can complement one another for function. Both T antigen and the host range mutant DBP have the ability to enhance adenovirus late gene expression in monkey cells. Furthermore, the defect in the DNA replication of the adenovirus DBP mutant Ad5*ts*125 at the nonpermissive temperature can be partially overcome by coinfection with WT, but not a T antigen *ts*A mutant of SV40. In return, coinfection with Ad2 helps SV40 *ts*A down-regulate the expression of its early genes (29, 55).

Genetic data suggest that, like SV40 T antigen, DBP contains separate functional domains, since there exist two classes of DBP mutations (temperature sensitive and host range), each of which affects very different activities and maps in a distinct region of the gene (3, 44–46). Analysis of Ad2*ts*400, the DBP mutant described in this paper, provides additional genetic and biochemical evidence that DBP contains separate functional domains. Ad2*ts*400, compared with WT Ad2, is a triple DBP mutant. It contains two host range alterations in the amino-terminal portion of the gene (the Ad2*hr*400 mutation and the cold-resistant mutation) that allow the virus to overcome the normal block to late gene expression in monkey cells, even at low temperature. In addition, Ad2*ts*400 contains a temperature sensitivity mutation in the carboxyl-terminal portion of the gene. The temperature-sensitive mutation causes a phenotype similar to that of the well-characterized DBP mutant Ad5*ts*125. Like Ad5*ts*125, Ad2*ts*400 is unable to replicate viral DNA or properly shut off early viral mRNA expression at the nonpermissive temperature.

Two sets of experiments suggest that the ability of the Ad2*ts*400 DBP to overcome the block to late gene expression in monkey cells is independent of its function in DNA replication. First, Ad2*ts*400 and Ad2 complement one another for growth in monkey CV_1 cells at the nonpermissive temperature. Presumably the Ad2 DBP supplies the DNA replication function, whereas the Ad2*ts*400 DBP allows normal late gene expression to occur. The simplest interpretation is that the ability of the Ad2*ts*400 DBP to allow late gene expression in monkey cells is not temperature sensitive. An alternative, although less attractive, explanation is that whereas this function is temperature sensitive, the Ad2*ts*400 DBP is stabilized in the mixed infection by interaction with WT DBP.

When this experiment was repeated in CV_c cells, a monkey cell line where the block to adenovirus late gene expression is more stringent, only partial complementation was observed. Incomplete complementation may be due to the WT DBP negatively interfering with late gene expression, although it is not clear why this does not occur in CV_1 cells. Perhaps mixed multimeric complexes consisting of WT and mutant DBP molecules are active in the CV_1 cellular environment, but are nonfunctional in the more restrictive CV_c cell.

To avoid problems inherent in the interpretation of such complementation experiments, temperature shift experiments were performed in which CV_c cells were infected with Ad2*ts*400 alone. Temperature shift-up during the beginning of the late phase of infection resulted in a rapid and dramatic inhibition of viral DNA replication, whereas the synthesis of the viral fiber protein (the expression of which is most dramatically affected in monkey cells) actually increased and then continued for at least 8 h. The most plausible interpretation of this result is that the late gene expression function of DBP is not disrupted at the nonpermissive temperature even though the DNA replication activity of this protein is destroyed. This interpretation, however, is somewhat clouded by our lack of understanding of the mechanism by which the altered DBP overcomes the monkey cell block to fiber synthesis. The DBP could presumably be acting at one or more levels of gene expression including transcription, RNA processing, RNA transport, and translation. If the late gene expression function of the Ad2*ts*400 DBP was in fact temperature sensitive, and the protein was acting primarily at the level of translation, we would expect to see an inhibition of fiber synthesis soon after repression of DNA synthesis occurred. This was not the case. If, on the other hand, the Ad2*ts*400 DBP was temperature sensitive for late gene expression, but was acting at the level of transcription, processing, or transport, we would expect a lag before inhibition of fiber synthesis occurred, since functional fiber mRNA made before the shift-up could continue to direct fiber protein synthesis. Nevertheless, a dramatic inhibition would be expected 8 to 12 h after the shift-up due to turnover of preexisting fiber messages, which have half-lives of approximately 2 h (K. P. Anderson and D. F. Klessig, unpublished data). This was not the case; the kinetics of fiber synthesis appeared identical to a control infection with Ad2*hr*405 (a non-temperature-sensitive derivative of Ad2*ts*400) in which DNA replication was prevented by araC treatment. Furthermore, fiber protein synthesis actually increased up to 8 h after the temperature shift-up. Such a result would not be expected if the cells were simply translating message made before the shift-up.

The results of the two sets of experiments taken together strongly suggest (i) that the DBP contains at least two functional domains, and (ii) that the domain that allows late gene expression in monkey cells can function independently of the domain involved in DNA replication. These functional domains may correspond to the two physical domains previously described (39, 60). The large C-terminal domain is known to possess the DNA binding and DNA replication activities of DBP (6, 39) and presumably also carries the activities that affect early gene shutoff and the efficiency of cellular transformation. Recent studies of revertants of Ad5*ts*125 and Ad5*ts*107 suggest that these C-terminal activities (DNA replication, shutoff of early genes, and modulation of cellular transformation) are themselves functionally distinct (13, 45, 48, 52, 53). In contrast, the smaller, heavily phosphorylated N-terminal domain presumably carries the

activity that allows normal late gene expression in monkey cells.

The cold-resistant mutation of Ad2*ts*400 defines a second class of Ad2 host range mutants that affect growth in monkey cells. The Ad2 host range mutants isolated previously, Ad2*hr*400 to 403, are all slightly cold sensitive for growth in monkey cells. We have shown that for Ad2*hr*400 this sensitivity is associated with a specific block to late gene expression, reminiscent of the block of WT Ad2 growth in monkey cells at 37°C. Ad2*ts*400 contains a second alteration in the N-terminal part of the DBP gene that allows the virus to express late genes and grow efficiently in monkey cells at low temperature. Preliminary characterization of Ad2*hr*405, a derivative of Ad2*ts*400 that no longer contains the temperature-sensitive mutation, but contains both host range mutations, shows that Ad2*hr*405 forms larger plaques than does Ad2*hr*400 at all temperatures, not just at 32.5°C. The cold-resistant mutation thus appears to enhance growth (and presumably late gene expression) in monkey cells regardless of temperature.

Since no such large-plaque-forming host range viruses have been isolated directly from Ad2 stocks, it seems likely that at least two DBP mutations are necessary for this phenotype. Sequence analysis of Ad2*hr*400 and the phenotypically similar host range mutants Ad2*hr*401 to 403, Ad5*hr*404, and Ad2$^+$ND3*hr*600 to 603 indicates that all contain an identical nucleotide alteration that leads to the substitution of tyrosine for histidine at amino acid 130 of the DBP (3, 46; D. Brough and D. Klessig, unpublished data). Characterization of the Ad2*ts*400 cold-resistant mutation shows that another change in this region of the DBP can affect host range. Since several additional cold-resistant mutants of Ad2*hr*400 have been isolated (D. F. Klessig, unpublished data), it should be possible to determine whether this second class of host range mutants is as restrictive as the original set of mutants with alterations at amino acid 130.

Since the biochemical basis of the block to late gene expression in monkey cells is not yet fully understood, the role played by DBP in overcoming the block cannot be clearly defined. Defects in late viral transcription, (K. P. Anderson and D. F. Klessig, unpublished data), mRNA processing (42; K. P. Anderson and D. F. Klessig, unpublished data), and translation (5) have all been observed in monkey cells abortively infected with human adenovirus. The presence in the cell of either the C-terminal portion of SV40 T antigen or an altered adenovirus DBP can alleviate these deficiencies and allow normal late gene expression. While T antigen and DBP probably overcome these blocks by the same mechanism, this awaits rigorous proof.

It seems likely that the N-terminal domain of DBP performs a vital function for viral infection in human as well as monkey cells, perhaps being required for normal late viral gene expression. The findings that adeno-associated virus requires an adenovirus early region 2A gene product for its growth (37, 58) and that Ad5*ts*125 is an efficient adenoassociated virus helper at the nonpermissive temperature (49) suggest such an N-terminal DBP activity in human cells.

The analysis of Ad2*ts*400 underscores the exquisite sensitivity of DBP to its cellular environment. In human cells, the WT DBP interacts properly with cell components to carry out its many functions. In cultured monkey cells, however, WT DBP presumably cannot interact correctly with cell components necessary for late viral gene expression, even though the putative cellular factors necessary for viral DNA replication are compatible with DBP. The Ad2*hr*400 mutation most likely alters DBP such that the interactions necessary for late gene expression occur efficiently at 37°C. At 32.5°C, however, an altered conformation of the Ad2*hr*400 DBP or monkey cell components (or both) may allow for only limited functional interaction. The efficiency with which the Ad2*hr*400 DBP can perform its late gene expression function(s) at 32.5°C seems to vary even between different lines of African green monkey kidney cells (Fig. 3b and 4). This cold-sensitive host range block, however, can be overcome by an additional DBP alteration, which presumably allows a functional interaction even at low temperature.

The interaction of DBP with host cell components is not limited to its function in late gene expression. For example, Younghusband et al. (71) found that Ad5*ts*125, but not WT virus, was defective for viral DNA replication in mouse cells at the permissive temperature. Further evidence for host interaction comes from studies of intragenic second-site revertants of Ad5*ts*125 and Ad5*ts*107 (45, 52, 53). One class of revertants is no longer temperature sensitive in HeLa cells, but remains temperature sensitive for growth in another human cell line, 293. Another class of revertants grows at 39.5°C in all cell types tested, but fails to autoregulate DBP synthesis in HeLa, but not CV_1 or 293, cells at this temperature. Clearly, DBP interacts with host cell components in carrying out many of its activities. Further analysis of this protein should provide insight into how it accomplishes its myriad of functions. Equally important, DBP should serve as a probe to identify the cellular factors involved in these complex and intriguing processess.

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