Deletion and Insertion Mutations in Early Region 1a of Type 5 Adenovirus That Produce Cold-Sensitive or Defective Phenotypes for Transformation

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On the basis of earlier findings showing that H5hr1 (hr1) is cold sensitive for transformation, a series of mutants were constructed so that they contained deletions or insertions in different sites of early region 1a (E1a) to ascertain: (i) whether the cold-sensitive phenotype of hr1 was the result of the identified single-base pair deletion of nucleotide 1,055 or due to a missense mutation at another site and (ii) what region and how much of the E1a 51-kilodalton protein is actually required to produce cell transformation. A mutant, H5dl101 (dl101), was constructed to contain a 5-base pair deletion of nucleotides 1,008 to 1,012, which produced a frameshift and a subsequent stop codon at nucleotide 1,241. This mutant, which should encode a truncated 33-kilodalton protein in place of the wild-type 51-kilodalton protein, had a cold-sensitive phenotype for transformation essentially identical to hr1. Consonant with this finding, a mutant (H5in106) engineered to contain a 16-base pair insertion initiated after nucleotide 1,009, with a stop codon beginning at the newly inserted nucleotide 1,013, also had a cold-sensitive phenotype like hr1 and dl101. It is striking, however, that a mutant (H5dl105) with a 69-base pair deletion beginning at nucleotide 1,003, and having a stop codon at nucleotide 1,544, was totally defective for transformation at any temperature. Transfection studies with plasmids containing the E1a or E1a and E1b genes of sub309, hr1, and dl101 further revealed that the cold-sensitive transformation phenotype observed could be exhibited in the absence of viral E1b gene expression.

The early region 1a (E1a) gene of adenovirus 5 (Ad5) (0 to 4.5 map units [MU]) has been shown to serve an essential role in initiating both the viral productive cycle in permissive cells (3, 24) and the transformation of rodent cells (15, 20, 43). Whereas a number of deletion, insertion, and point mutations have been introduced into this gene (7, 17, 25, 39), very little is known about the mechanism(s) by which E1a-encoded proteins may interact with cellular factors (31, 32) or virus-encoded macromolecules or both to elicit their biological effects.

During the viral productive infection two spliced mRNAs are synthesized early, 12S and 13S (4, 10, 26, 41), and Montell et al. (30) have shown that the proteins encoded by the smaller 12S mRNA are not obligatory for viral replication. Viral host range mutants containing alterations in the 13S mRNA coding capacity have thus far been shown to be defective in transformation and viral replicative functions, which suggests that the acidic 51-kilodalton (kd) protein encoded by this mRNA (35) plays a critical role in both processes.

The requirement for the 51-kd protein in the process of cellular transformation has been demonstrated by using a viral host range mutant, hr1 (21), which produces a truncated form of this protein (35). Recent investigations indicate that hr1 is cold sensitive for the maintenance of transformation (1, 22). By using a cloned rat embryo fibroblast cell line, CREF, it was demonstrated that clones of hr1-transformed cells display the transformed phenotype in a temperature-dependent manner; i.e., at 32°C they reflect the normal phenotype of CREF cells and when shifted to 37°C as long as 3 weeks after infection they convert to the transformed phenotype (1). These observations in conjunction with DNA

transfer-filter hybridization studies suggest that those events leading to initiation, i.e., integration, are not conditionally regulated in hr1-infected cells.

Although it has been demonstrated that hr1 contains a single-base pair deletion at nucleotide 1,055 (35), the entire sequence of the E1a gene was not determined. Since this mutant was isolated with nitrous acid, it was possible that an additional missense mutation was present and that this mutation produced the conditionally lethal phenotype. To approach this problem and to probe the function(s) of the 51-kd protein further, three Ad5 mutants were constructed so that they contain deletions or insertions of nucleotide sequences around the *Sma*I restriction endonuclease cleavage site (2.8 MU). Two of the viral mutants exhibited a cold-sensitive transformation phenotype which was similar to that found with hr1 and confirmed that the single-base pair deletion in hr1 was in fact responsible for the observed phenotype. In addition, DNA sequence analyses of the altered viral E1a gene sequences from the three viral mutants revealed that coding sequences varying by no more than 5 base pairs (bp) could determine whether a truncated protein produced by the 13S mRNA was conditionally functional or inactive at 32 and 37°C. DNA transfection studies described in this paper demonstrate that the E1a gene sequences from the mutant viral genomes are capable of eliciting the conditional phenotype in the absence of E1b gene functions.

MATERIALS AND METHODS

Construction and characterization of Ad5 E1a deletion and insertion mutants. The *Hpa*I-E DNA fragment of Ad5 (0 to 4.5 MU) was cloned into the *Pst*I site of pBR322 (5), using the procedure described by Stow (42). A 2-μg portion of this

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FIG. 1. Diagram of the methods used to construct mutants in the E1A region of Ad5. The diagram shown represents the construction of dl101 and dl105 by ligation of the mutated 0- to 3.8-MU fragment to the 3.8- to 100-MU fragment of sub309 and the formation of in106 by overlap recombination between the mutated 0- to 4.5-MU fragment and the 3.8- to 100-MU fragment of sub309. The 0- to 4.5-MU DNA fragment of Ad5 was cloned into the *Pst*I site in pBR322. Open parentheses represent areas of deleted DNA sequences, and open triangles denote restriction enzyme cleavage sites present on the viral genomes.

plasmid DNA was cleaved with *Xma*I (2.8 MU) and digested with either 6.25 U or nuclease S1 (P-L Biochemicals) for 30 min at 30°C or 0.001 U of nuclease *Bal*31 (Bethesda Research Laboratories) for 30 min at 37°C. After the recircularization of these molecules with T4 ligase and cleavage with *Sma*I to remove any molecules that escaped nuclease digestion, *Escherichia coli* HB101 cells were transformed to tetracycline resistance. Plasmids containing the desired deletions were isolated, and their DNAs were purified by the Sarkosyl-lysate procedure of Clewell and Helinski (11).

To prepare insertions of linker DNA into the *Sma*I restriction enzyme site (Fig. 1), 1 µg of plasmid DNA was digested with this enzyme and ligated with a 200-fold excess of *Xba*I linker ends (CTCTAGAG; Collaborative Research). After digestion with *Sma*I, the ligated molecules were used to transform *E. coli* HB101 cells, selecting for tetracycline-resistant colonies containing plasmids with two *Xba*I sites 2.8 and 3.8 MU.

The modified viral E1a gene contained within these plasmids were introduced into the viral genome of sub309 (25) by a procedure described by Stow (42) for dl101 and dl105 or by overlap recombination (9). To enhance the frequency of the overlap recombination event, subconfluent cultures of 293 cells (19) maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum were exposed to 15 J of UV light (254 nm; General Electric germicidal lamp), and the irradiated cells were then incubated for 16 h at 37°C in the dark. After the transfection of these cells (19) with the overlapping DNAs (1 µg of sub309 DNA cleaved with *Cla*I and *Xba*I and 1 µg equivalent of plasmid DNA containing E1a gene sequences cleaved with *Pst*I), viral plaques were isolated after incubation for 11 days at 37°C.

All viruses were plaque purified three times before producing stocks for characterization and use. Mutant viruses

were identified by their *Sma*I digestion pattern (Fig. 2), which revealed the loss of one enzyme site at 2.8 MU. The size of the deletions generated and the position of *Xba*I linker in in106 was determined by cloning the *Cla*I (2.6 MU)-*Hpa*I (4.5 MU) DNA fragment from each virus into plasmid vector pDR33 (37) containing unique *Cla*I and *Hpa*I sites. All of the plasmids constructed were propagated in *E. coli* strain GM33 (27), which is deficient in adenine methylase. The DNA was sequenced, according to the Maxam and Gilbert (28) technique, after the plasmids were linearized at the *Cla*I site, and the 5' end of the plasmid DNA was phosphorylated with T4 polynucleotide kinase.

Viral transformation and DNA transfection assays with CREF cells. CREF cells were used for viral transformation assays as previously described (1, 12) at a multiplicity of 10 PFU of H5sub309, H5dl101, or H5in106 or 30 PFU of H5dl105 per cell. DNA transfection assays were performed with 7 µg of recombinant plasmid DNA, with 3 µg of salmon sperm DNA as carrier (19). Approximately 10^6 CREF cells in monolayers in 10-cm plates were transfected, and after 4 h at 37°C the plates were treated successively with 15% glycerol in phosphate-buffered saline, plain phosphate-buffered saline alone, and trypsin (0.125%)-EDTA (0.05%). The cells were replated at 10^5 cells in a 5-cm culture dish and fed with low- Ca^{2+} medium twice a week for 8 weeks at 37°C or 11 weeks at 32°C, after which transformed foci were counted and isolated. In all assays, each group contained eight cultures of replated cells (i.e., 10^5 cells per culture dish).

Construction of recombinant plasmids containing early gene sequences from hr1, dl101, and sub309. The steps involved in the cloning of the E1a gene (*Hpa*I-E fragment, 0 to 45 MU) or E1a and E1b genes (*Xho*I-C fragment, 0 to 15.5 MU) of hr1, sub309, and dl101 are outlined and described in Fig. 3. pLB209 was constructed with pACYC177 plasmid DNA (8)

FIG. 2. Electrophoretic analysis of mutant viral genomes, using *SmaI* and *XbaI* restriction endonucleases. A 1- μ g amount of DNA from each virus described was digested with 4 U of (A) *SmaI* (sub309, dl101, dl105, and in106) or (B) *XbaI* (sub309 and in106), and the fragments were separated on a 0.6% agarose gel containing 1 μ g of ethidium bromide per ml. The bands were visualized with UV light and photographed. The F/K* band generated by *SmaI* cleavage of dl101, dl105, and in106 represents a fusion of the *SmaI*-F + K bands since the enzyme site at 2.8 MU is no longer present. The presence of an additional *XbaI* site at 2.8 MU is detected by cleavage of in106 with this enzyme to yield two unique DNA fragments, B1 (0 to 2.8 MU) and B2 (2.8 to 3.8 MU). The DNA fragment labeled B in the sub309 lane extends from 0 to 3.8 MU.

and the Ad5 *XhoI*-C fragment instead of the Ad2 *XhoI*-C fragment by a procedure previously described (2). All DNA fragments used for ligation and transformation were purified with low-melting-point agarose (Bethesda Research Laboratories). For the dl101 plasmids PLB211 and PLB214, the presence of the deletion at 2.8 MU was confirmed by restriction enzyme analysis.

Characterization of cells transformed by dl101, hr1, in106, and sub309. Two transformed foci resulting from viral infection or DNA transfection from each virus type were cloned by seeding 100 cells per 90-mm plate and isolating well-separated clones by using steel cloning cylinders. The ability of the cloned cell lines to grow in an anchorage-independent manner was assayed in agar as previously described (13). Briefly, 10^3 , 10^4 , or 10^5 cells in low- Ca^{2+} medium supplemented with 0.4% Noble agar were layered on 0.8% agar bases prepared in low- Ca^{2+} medium. The plates were fed once a week with 3 ml of 0.4% agar in low- Ca^{2+} medium, and colonies were counted at 3 weeks for 37°C and 5 to 6 weeks for 32°C cultures.

The presence of viral or plasmid DNA sequences in the cloned transformed cell lines was determined by DNA filter-transfer hybridization analyses, as previously described (12, 36, 40, 45).

RESULTS

Construction and isolation of viral deletion and insertion mutants. The protocol used to construct Ad5 insertion and deletion mutants so that the coding capacity of the E1a 13S

mRNA was affected is described in Fig. 1. Briefly, a plasmid containing a cloned E1a gene (0 to 4.5 MU) of Ad5 was linearized with *XmaI* and digested with nuclease S1 or *Bal31*. By using an approach described by Stow (42), the modified viral E1a gene sequences were introduced back into a complete viral genome, using mutant H5sub309 (sub309) DNA (24). sub309 virus has a phenotype similar to that of wild-type Ad5 (Ad5wt), but structurally it contains only one *XbaI* site at 3.8 MU. Because of this genomic organization, the 3.8- to 100-MU fragment of this sub309 can be isolated and ligated to the 0- to 3.8-MU mutated DNA fragments derived from the isolated plasmids. These ligated molecules were then used to transfect 293 cells in a direct plaque assay.

Alternatively, *SmaI*-digested plasmid DNA was ligated to the octanucleotide CTCTAGAG, which contained an *XbaI* recognition site. The E1a sequences containing a newly inserted *XbaI* site at 2.8 MU were then introduced into sub309 DNA via an overlap recombination mechanism (9). However, the small area of homology existing between the two overlapping DNA fragments (250 bp) resulted in a very low frequency of recombination. To enhance the occurrence of the recombinational event, 293 cells were pretreated with UV light and transfected after a 16-h induction period in the dark. The UV irradiation effected an enhanced ability to recombine the overlapping DNA fragments upon transfection (L. E. Babiss and H. S. Ginsberg, manuscript in preparation).

To confirm that the viral plaque isolates obtained did contain alterations in their E1a gene sequences, after two cycles of plaque purification on 293 cells, viral DNA was isolated and analyzed by using *SmaI* or *XbaI* restriction endonuclease. The three viral mutants analyzed all contained alterations at 2.8 MU, which prevented *SmaI* cleavage at that site (Fig. 2A). As a result, the *SmaI*-K and -F fragments present in the parental sub309 virus were fused in the mutants viruses and resulted in a new DNA band of higher molecular weight. H5in106 (in106) was also found to contain an additional *XbaI* site at about 2.8 MU, which confirmed the presence of the linker molecule within the viral genome (Fig. 2B).

To determine whether a frameshift mutation had occurred as a result of the deletions introduced at 2.8 MU or whether the linker sequences ligated properly at the site, DNA sequence analyses were performed (28). By using a cloned DNA fragment derived from each of the mutant viral genomes, it was determined that H5dl101 (dl101) contained a 5-bp deletion extending from 1,007 to 1,013 bp; H5dl105 (dl105) contained a 69-bp deletion extending from 1,002 to 1,072 bp; and H5in106 contained an insertion of 16 bp between 1,009 and 1,010 bp (Fig. 4). Although no alteration of the reading frame occurred for dl105, the frameshift mutation that was introduced into the coding region of the 13S mRNA for dl101 resulted in the occurrence of a stop codon beginning at nucleotide 1,241 and presumably caused termination of translation. The 16-bp insertion determined for in106 indicated that two linker molecules were ligated into the *SmaI* site in tandem, which produced an in-frame stop codon at the inserted nucleotide 1,013.

Transformation characteristics of sub309 and E1a deletion and insertion mutants. It was previously shown that hr1 exhibits a cold-sensitive phenotype for transformation, using both primary rat embryo cells (22) and CRE6 cells (1). When CRE6 cells were infected with dl101, followed by replating of the infected cells at 32°C and growth for 6 weeks at 32°C, morphologically transformed foci could not be discerned

FIG. 3. Methods for constructing plasmids containing mutations in the early regions of Ad5 DNA. The procedure for cloning the E1a and E1b genes of each virus indicated is presented (A). pLB209 contains the *XhoI*-C (0 to 15.5 MU) fragment of Ad5 cloned into pACYC177 (8) and its construction has been described before (2). A 2- μ g amount of hr1, sub309, or dl101 virus DNA was cleaved with *ClaI* and *XhoI* and mixed and ligated with 100 ng of pLB209 cleaved with the same enzymes and *HpaI* to inactivate the Ad5wt fragment extending from 2.6 to 15.5 MU. After transformation of *E. coli* HB101 cells, ampicillin-resistant colonies were selected and the resulting plasmids, pLB210, pLB211, and pLB212, were isolated. To clone only the E1a gene of each virus (B), one μ g of each plasmid described above was cleaved with *BamHI* and *HpaI* and ligated to 100 ng of pDR33 plasmid DNA cleaved with the same enzymes and *Sall*. Ampicillin-resistant colonies were selected after transformations of *E. coli* HB101 cells, and plasmids pLB213, pLB214, and pLB215 were isolated.

(Table 1). However, like hr1, if dl101-infected CREF cells were maintained at 37°C, the frequency of transformation was fivefold higher than for sub309 or Ad5wt-infected cultures (Table 1). It should also be noted that dl101 and in106 (described below), like hr1, had the same minimal cytopathic effects on CREF cells as sub309, and thus the increased transformation frequency produced by these mutants must be attributed directly to the altered viral product.

All foci generated by the mutant virus at 37°C exhibited a fibroblastic morphology; in contrast, sub309 and Ad5wt foci contained a mixture of both fibroblastic and epithelioid colonies. Shift-up and shift-down experiments with dl101-infected CREF cells were similar to the previous findings with hr1 (1). When mutant-infected cell cultures were shifted from 32 to 37°C 2 or 3 weeks after infection, the frequency of transformation approached that of infected cells maintained at 37°C throughout the experimental period (Table 1). Con-

versely, upon shifting cultures from 37 to 32°C, the number of foci decreased, the majority of the transformed foci became flattened, and the cells were not dissimilar from the surrounding CREF cells.

It is important to note that the cold-sensitive nature of these viruses is not absolute. CREF cells infected with hr1 or dl101, and maintained at 32°C for a period of 8 weeks, could develop a few small foci, which probably reflected a small amount of "leakiness" associated with the diminished function of the truncated protein.

From these observations it can be inferred that hr1 probably does not contain a second-site mutation. In addition, the functional domain of the 51-kd protein could now be more precisely positioned since the mutation present in dl101 was 47 bp upstream from the hr1 deletion.

To determine whether the additional missense amino acids present in the truncated protein encoded by dl101 were

FIG. 4. Nucleotide sequences of deletions and insertions constructed in the 13S mRNA of the E1a region of Ad5 and proposed translation product. A DNA fragment extending from 2.6 (*Clal*) to 4.5 (*HpaI*) MU from dl101, dl105, and in106 was cloned into plasmid vector pDR33 and was propagated in a dam strain of *E. coli* (GM33). The DNA sequence from the *Clal* site was determined by the chemical degradation method of Maxam and Gilbert (28). The sequence data presented for Ad5wt and hr1 were determined by van Ormondt et al. (44) and Ricciardi et al. (35), respectively. Dashed lines represent sequences removed during synthesis of the 13S mRNA. Hatched boxes show the deleted (Δ) DNA sequence in each mutant, and the bold line above the sequence indicates insertions (∇) of linker DNA. Parentheses around the protein molecular weight (M.W.) values indicate predicted sizes from DNA sequence data (44).

acting to stabilize it in a conditionally active conformation at 37°C, a mutant virus was constructed, in106, which contained a 16-bp insertion at 2.8 MU and generated an in-frame translational termination signal beginning at the newly inserted nucleotide 1,013 (Fig. 4). The position of this stop codon enables the 13S mRNA to encode a predicted truncated protein of 24 kd, containing the same amount of sense amino acids as dl101 but only one missense amino acid.

Transformation studies performed using in106 with CREP cells revealed that it had a conditional, cold-sensitive phenotype similar to that of dl101 (Table 1). This indicated that the missense amino acids present in the truncated protein encoded dl101 were not acting to stabilize it in a conditionally active form.

As a means of more accurately determining what domain of the 51-kd protein was essential to retain some function, dl105 virus (Fig. 4) was used to infect CREP cells in a transformation assay at 32 and 37°C. Upon infection with as much as 30 PFU/cell, no discernible foci developed at either temperature for incubation periods extending up to 8 weeks (Table 1). The 69-bp deletion asymmetrically located around 2.8 MU distinguished dl105 from dl101 in two ways. First, the sense amino acids incorporated downstream from the deletion present in dl105 appeared unable to influence the function of the truncated protein. Second, the coding sequences upstream from the start of the deletions present in both dl101 and dl105 revealed a difference of only five nucleotides. As a consequence, the truncated protein encoded by dl105 contained two fewer sense amino acids (codons GAG and CAC at positions 148 and 149; refer to Table 2). Therefore, the nucleotide difference between the function of a protein active at 37°C and a protein that cannot produce transformation at either temperature could be localized to a 5-bp region extending from nucleotides 1,002 to 1,007.

Biological properties and integration patterns of mutant

TABLE 1. Comparative transformation of CREP cells by Ad5wt, sub309, and E1a deletion and insertion mutants at various temperatures

Expt temp (°C) ^a	Incubation period (wk)	Transformation	
		Foci per plate (mean $\pm$ SD) ^b	Frequency
Ad5wt			
32	6	26 $\pm$ 2	2.6 $\times$ 10 ⁻⁴
37	6	24 $\pm$ 3	2.4 $\times$ 10 ⁻⁴
sub309			
32	6	29 $\pm$ 5	2.9 $\times$ 10 ⁻⁴
37	6	35 $\pm$ 3	3.5 $\times$ 10 ⁻⁴
dl101			
32	6	0	<1 $\times$ 10 ⁻⁵
37	6	179 $\pm$ 7	1.79 $\times$ 10 ⁻³
32 $\rightarrow$ 37	2 + 4	149 $\pm$ 6	1.49 $\times$ 10 ⁻³
32 $\rightarrow$ 37	3 + 3	85 $\pm$ 2	8.5 $\times$ 10 ⁻⁴
37 $\rightarrow$ 32	2 + 4	7 $\pm$ 2	7 $\times$ 10 ⁻⁵
37 $\rightarrow$ 32	3 + 3	42 $\pm$ 7	4.2 $\times$ 10 ⁻⁴
dl105			
32	8	0	<1 $\times$ 10 ⁻⁵
37	8	0	<1 $\times$ 10 ⁻⁵
in106			
32	6	0	<1 $\times$ 10 ⁻⁵
37	6	163 $\pm$ 5	1.6 $\times$ 10 ⁻³

^a CREP cells were infected with 10 PFU of Ad5wt, sub309, dl101, or in106 or with 30 PFU of dl105 per cell; after 2 h of viral adsorption at 32°C, cells were resuspended and reseeded at 10^5 cells per 60-mm plate at 32 or 37°C. Cultures were either maintained at 32 or 37°C continuously for 6 to 8 weeks or shifted to the indicated temperature at 2 or 3 weeks postinfection. All cultures were fixed and stained at the times indicated.

^b Mean number of transformed colonies or foci per 10^5 cells. Each group consisted of eight culture plates.

TABLE 2. Summary of DNA sequences, proposed amino acid positions, and transformation data of Ad5wt and E1a mutant viruses

Virus	Mutation		Amino acid at position ^a :					Transformation	
	Type (bp) ^a	Site	148	149	150	151	152	Foci ^b	Cold sensitive
Ad5wt			glu	- his	- pro	- gly	- his	+	-
5hr1	Δ1	1,055	glu	- his	- pro	- gly	- his	+	+
dl101	Δ5	1,007-1,013	glu	- his	- pro	- arg	- leu	+	+
in106	▽16	1,009-1,010	glu	- his	- pro	- leu	- stop	+	+
dl105	Δ69	1,002-1,072	asp	- ser	- leu	- cys	- tyr	-	-

^a Δ, Deletion; ▽, insertion. Amino acids and location were not determined experimentally, but were inferred from the nucleotide sequence of this gene by van Ormondt et al. (44) and the deletion and insertion mutants presented in Fig. 4.

^b Ability to induce focus formation at 37°C.

virus-transformed CREF cells at 32 and 37°C. The patterns of viral DNA integration into chromosomal DNA of two sets of cloned dl101- and in106-transformed CREF cell lines were examined by cleavage with *Xba*I or *Bgl*II followed by DNA transfer-filter hybridization analysis (40). Cell line 101-6 appeared to contain dl101 genome DNA integrated at several sites (Fig. 5A), whereas 101-8 appeared to contain a single viral DNA integration site. *Bgl*II cleavage of cellular DNA isolated from cell lines 106-B2 and 106-B4 revealed that most of the viral genome was present in these cells, with the terminal fragments being larger owing to integration (Fig. 5B). Like hr1-transformed CREF cell lines (1), the integration pattern of the cloned cells was not altered by shifting the cultures to 32°C and maintaining them at that temperature (data not shown).

Whereas the transformation studies showed similarities among hr1, dl101, and in106, it was important to determine

whether the cloned cell lines derived from transformed cell foci produced by the latter two viruses continued to exhibit the cold-sensitive phenotype. Both cell lines 101-6 and 101-8 at 37°C had cloning efficiencies in agar that were similar to or exceeded that of wt3A, an Ad5wt-transformed CREF cell line (12) (Table 3). When assayed at 32°C, the cloning efficiency of wt3A remained relatively unchanged, but cell lines 101-6 and 101-8 were decreased by 73 and 95%, respectively. Whereas the sizes of the growing colonies of wt3A cells at 32 and 37°C were similar, there was a marked decrease in the colony sizes of mutant virus-transformed cell colonies grown in 32°C when compared with those incubated at 37°C. In addition, the high agar cloning efficiency of cell line 101-6 at 37°C in comparison with wt3A, 101-8, and 106-B4 may be due to an enhanced level of viral gene expression, perhaps as a result of the integration of a greater number of genome copies (Fig. 5A).

Construction of plasmids containing the E1a or E1a and E1b genes from dl101, hr1, and sub309. The studies described above strongly indicated that the deletion present in the E1a gene of hr1 was in fact the cause of the conditional transformation phenotype and that a region around 2.8 MU was critical for the function of the 51-kd protein. It remained to be determined whether the cold-sensitive nature of the truncated proteins encoded by the mutant viruses required functions of viral E1b gene products or was possibly due to an interaction of the E1a protein with some cellular factor(s). To test these possibilities, plasmids were constructed which

FIG. 5. Analysis of dl101 and in106 sequences in CREF cells. A 10-μg portion of cellular DNA isolated from the cloned cell lines indicated was cleaved with *Xba*I (A) or *Bgl*II (B), and the fragments were separated by electrophoresis in 0.6% agarose, transferred to nitrocellulose sheets by blotting, and hybridized to sub309 DNA labeled with ³²P by nick translation (36, 40, 45). The marker lane in (A) represents five genome equivalents of sub309 DNA cleaved with *Xba*I, and the marker lane in (B) represents the same quantity of sub309 DNA cleaved with *Bgl*II. The starred DNA bands represent terminal fragments which are not present in the transformed cell lines due to viral DNA integration.

TABLE 3. Cloning efficiency in soft agar of uninfected, wt5-transformed, dl101-transformed, and in106-transformed CREF cells grown at various temperatures

Cell line	Cloning efficiency in agar (%) ^a	
	37°C	32°C
CREF	<0.001	<0.001
Ad5wt-3A	45 ± 2	38 ± 2
dl101-6	98 ± 1	27 ± 2 ^b
dl101-8	47 ± 5	3.2 ± 1 ^b
in106-B4	53 ± 8	9 ± 2 ^b

^a Approximately 10³, 10⁴, or 10⁵ cells were prepared in low-Ca²⁺ medium containing 0.4% Noble agar and seeded at 37 or 32°C on 0.8% agar base layers prepared in the same medium. Plates were fed once a week with 2 to 3 ml of 0.4% agar on low-Ca²⁺ medium, and colonies were counted after 3 weeks for 37°C and after 5 to 6 weeks for 32°C cultures. Each value is the mean ± standard deviation of four plates.

^b The final sizes of the mutant virus-transformed cell colonies counted at 32°C were consistently four to five times smaller than the colonies cultured at 37°C.

TABLE 4. Transforming activity in CREF cells of hybrid plasmids containing E1a or E1a and E1b gene sequences of dl101, hr1, and sub309

Plasmid clone used for transformation ^a	Cloned viral DNA		Foci per plate (mean $\pm$ SD) ^b	
	Origin	Genome region	32°C	37°C
pLB210	sub309	E1a + E1b	8 $\pm$ 2	8 $\pm$ 2
pLB211	dl101	E1a + E1b	0	19 $\pm$ 3
pLB212	hr1	E1a + E1b	0	22 $\pm$ 1
pLB213	sub309	E1a	6 $\pm$ 2	8 $\pm$ 1
pLB214	dl101	E1a	0	10 $\pm$ 1
pLB215	hr1	E1a	0	6 $\pm$ 1
pACYC177			0	0

^a Approximately 10^6 CREF cells were transfected with 7 μ g of the desired plasmid DNA, using the calcium precipitation technique. After 3 h of incubation at 37°C, 15% glycerol in phosphate-buffered saline was added, after which cells were resuspended and reseeded at 10^5 cells per 60-mm plate at 32°C for 11 weeks or at 37°C. Cultures were maintained at 32°C for 11 weeks or at 37°C for 8 weeks, at which time the monolayers were fixed, stained, and counted. A 7- μ g portion of E1a-containing plasmids equals 59- μ g genome equivalents; 7 μ g of E1a + E1b-containing plasmids equals 31- μ g genome equivalents.

^b Mean number of transformed colonies per 10^5 cells.

contained the DNA sequences of either E1a or E1a and E1b from dl101, hr1, and sub309, and the recombinant plasmids were used to transfect CREF cells to note their transformation potentials at 32 and 37°C.

The construction of the various hybrid plasmids containing E1a or E1a and E1b gene sequences is described in Fig. 3. Mutant viral E1a and E1b DNA sequences (2.6 to 15.5 MU) were substituted for comparable wt sequences to generate an intact viral sequence extending from 0 to 15.5 MU. With this approach, a series of plasmids were isolated containing the E1a and E1b regions of the desired viral genomes. The newly isolated plasmid strains were used as a source for viral E1a sequences (0 to 4.5 MU), which were subcloned into plasmid vector pDR33 (Fig. 3B).

Plasmid DNAs were used to transfect CREF cells in a transformation assay at 32 and 37°C. All of the plasmids containing viral DNA sequences were capable of producing transformed foci at 37°C (Table 4). However, no foci appeared at 32°C when any of the plasmids derived from hr1 or dl101 were used. In contrast, sub309 plasmids transformed CREF cells at the same frequency at 32 or 37°C. Therefore, the viral transformation studies described above correlated exactly with the results of the plasmid transfection experiments.

It should be noted that the morphology of E1a- and E1a plus E1b-transformed CREF cells were different. Transformed foci produced by E1a plasmids (pLB213–pLB215) were fibroblastic and relatively flat. In contrast, sub309 E1a plus E1b plasmid-transformed CREF cells were a mixture of fibroblastic and epithelioid cells, and the cell colonies were raised. However, foci produced at 37°C by plasmids containing both E1a and E1b DNA from hr1 or dl101 (pLB211 plus pLB212) were solely fibroblastic but retained the ability to produce raised colonies. DNA filter-hybridization studies demonstrated that transformed CREF cells, which had been transfected with the various plasmid DNAs, contained the expected viral DNA sequences (data not shown). In addition, the ability of the cloned transformed cell lines to grow in agar correlated with their ability to express the cold-

sensitive phenotype: the E1a- and E1a plus E1b-transformed cells derived from hr1 or dl101 showed reductions in agar cloning efficiencies when grown at 32°C as opposed to 37°C (data not shown).

DISCUSSION

The data presented in this paper demonstrate that when frameshift mutations are introduced into the 13S mRNA of the E1a gene of Ad5 via deletion or insertion of genetic material, some of the viral mutants obtained exhibit a conditional, cold-sensitive transformation phenotype. This phenotype can be attributed solely to the altered gene product encoded in the 13S mRNA since the defects were placed in the introns of the 12S and 9S mRNA transcripts, thus leaving these mRNAs, and hence their gene products, unaltered. The initial viral mutant isolated, dl101, was engineered to contain a 5-bp deletion at 2.8 MU (nucleotides 1,007 to 1,012), and it was utilized to investigate: (i) whether hr1 might contain a missense mutation, in addition to the single-base pair deletion, to account for its conditionally lethal, cold-sensitive phenotype; and (ii) how much of the 51-kd E1a protein was required to transform cells.

Previous studies had shown that the truncated 28-kd protein encoded by the 13S mRNA of hr1 (35) effected the conditionally functional phenotype for the maintenance of the transformed cell (1, 22). However, it was necessary to determine whether a mutation other than the known single-base pair deletion in hr1 was producing the conditional phenotype observed since it is uncommon for a nonsense mutation to display a conditionally lethal phenotype. Whereas marker rescue (14) of the known mutation or back crossing of the mutation into a known wt genome is the traditional approach to the problem, dl101 answered this question by revealing that a frameshift mutation introduced 47 bp upstream from the lesion present in hr1 could generate the same phenotypic characteristics. Unlike hr1, dl101's mutation was introduced via in vitro manipulation and therefore decreased the likelihood of there being an additional mutation upstream from the engineered deletion.

It is important to note that the cold-sensitive mutants described in this communication did not exhibit a normal transformed phenotype even when maintained at the permissive temperature of 37°C. Thus, all of the cells transformed by hr1, dl101, and in106 at 37°C were fibroblastic, as opposed to epithelioid, and they were generated at a five-fold-higher frequency than was wt virus. These data indicate that at least one domain of the truncated protein produced by these viruses may be conditionally functional and that, when this domain(s) was reduced by as few as five nucleotides, it had lost its ability to function normally at either temperature. It was previously shown that hr1-transformed cells grown at 32 or 37°C maintain the same viral DNA integration pattern (1) and that hr1- or dl101-infected CREF cells shifted after prolonged periods at 32 to 37°C still produce transformed cell foci. These findings suggest that those events that lead to the integration of the viral genome into the host cell chromosome, and possibly the initial expression of E1a gene products, are unaffected by the mutation present or even enhance the ability of the mutant virus to initiate these events. Studies with these viruses are presently under way to determine whether an increase in the overall frequency of integration is the event responsible for the higher transformation frequencies observed.

The position of the deletion present in dl101 further suggested that only a limited region of the 51-kd protein was

required to retain partial functionality. However, since this mutant did not encode an in-frame stop codon (TGA) until nucleotides 1,241 to 1,243, it was possible that the additional missense amino acids incorporated into the truncated protein were functioning to stabilize it in a conditionally active form. This problem was approached by constructing a mutant virus, in106, so that it contained a 16-bp insertion between nucleotides 1,009 and 1,010, which generated an in-frame stop codon (TAG) at the newly created position 1,113 to 1,116 (Fig. 4). Transformation studies with this virus revealed that it behaved like hr1 and dl101 and thus demonstrated that the missense amino acids present in the truncated protein encoded by dl101 and absent in the protein encoded by in106 were not required to retain the conditional transformation functions observed for either virus.

DNA sequence analysis of dl105 revealed that it contains a 69-bp deletion extending from nucleotides 1,002 to 1,072. Unlike all of the previous viral mutants tested, this virus was completely incapable of generating transformed CREF cell foci at multiplicities as high as 30 PFU/cell regardless of the temperature of incubation. Since the deletion present in dl105 was not symmetrical around the *Sma*I restriction endonuclease cleavage site at 1,009 bp, this mutant and dl101 only differed by 5 bp upstream from the start of the deletion. The mutants' characteristics (Table 2) note that wt5, hr1, dl101, and in106 retain the same amino acid sequence as wt virus at positions 148 to 150, at which site missense amino acids are introduced. Whereas dl101 was shown to contain a 5-bp deletion, the first amino acid produced owing to the frameshift remained a proline at position 150 due to degeneracy of the amino acid code. Whether this histidine-proline amino acid sequence is absolutely required for the truncated proteins produced by these mutant viruses to function in transformation, or possibly to maintain its integrity, is presently being investigated by constructing an additional viral mutant with a 4-bp deletion at this site. This type of lesion would result in an amino acid sequence of histidine and arginine at positions 149 and 150, respectively. It is important to note that, although H5in500 (7) retains the histidine-proline amino acids, it is transformation defective just as are all of the previously isolated viral mutants that do not retain this amino acid sequence (25, 39). However, the low frequencies of transformation reported by these investigators, perhaps owing to the different rat cells used, may not have allowed them to detect the partial transformation phenotype that is obtained with the E1a mutants described in this communication.

Ho et al. (22) described the isolation of Ad5 mutants that are cold sensitive for replication in HeLa cells. Both dl101 and in106 also display this type of conditionally host range lethal phenotype for replication (manuscript in preparation). However, although the final viral yield obtained in HeLa cells with either virus at 37°C approaches that of Ad5wt, the eclipse period is extended, suggesting that in replication as in transformation only a partial function of the truncated protein is attained at this temperature.

The lesion that produces a conditional phenotype is classically a missense mutation at a single base pair. Although our observations that frameshift mutations brought about by deletion or insertion of genetic material can yield a cold-sensitive phenotype are unusual, they are not unique to this system. Pintel et al. (34) isolated a deletion mutant of simian virus 40 which lacks 81 bp at the C-terminal end of the large T antigen and demonstrated that transformation of rat cells by this virus was cold sensitive. These findings were further

extended (33) to show that this virus was heat sensitive for viral replication, whereas, in contrast, the transformed cells derived from this virus were cold sensitive for maintenance of the transformed phenotype. Bryant and Parsons (6) have constructed deletion mutants of Rous sarcoma viruses and have shown that one mutant, tsCH119, is heat sensitive for the maintenance of the transformed phenotype in chicken cells.

It has previously been shown that the E1a gene of Ad5 (23) or Ad12 (38) can partially transform rodent cells, whereas studies that used a DNA fragment from Ad5 extending from 0 to 8.0 MU revealed that E1a and partial E1b gene products (the 19K and truncated 58K proteins) can effect complete transformation (16). Those findings suggest that a E1a gene product(s) may act independently or interact with cellular factors to initiate and maintain the partial transformed phenotype, which also has been a proposed function for the E1a gene product(s) in viral replication (31, 32). The expression of the E1b-encoded 19K and truncated 58K proteins may produce the completely transformed cells observed by their interacting with E1a proteins or cellular factors or both, or they may function independently.

To determine whether the cold-sensitive phenotype could be expressed in the absence of E1b gene products, recombinant plasmids were constructed which contained the E1a or E1a and E1b genes of sub390, hr1, and dl101, and these were used to transfect CREF cells in a transformation assay. Although it has been demonstrated that the processes leading to cell transformation by infection or transfection of susceptible cells can vary (29), our transfection studies correlated completely with the results of virion infections (Table 4). The transformation frequencies with plasmids containing E1a or E1a and E1b gene sequences from hr1 and dl101 were higher at 37°C than those with plasmids derived from sub309, in keeping with the increased transformation frequency noted when the mutant virions were used. In addition, the morphology of the transformed cells obtained with the mutant viruses or sub309 E1a gene-containing plasmid was fibroblastic in contrast to the epithelioid cells produced by the sub309 plasmids containing E1a and E1b. E1a gene sequences transfected into CREF cells derived from hr1 or dl101 were unable to induce foci at 32°C, indicating that they had retained their conditional phenotype in the absence of viral E1b gene expression.

The data described using viruses with specific mutations in the portion of the genome encoding the E1a 51-kd protein imply that it is this viral gene product that is necessary to maintain the recognized properties of transformed cells. The conditionally lethal, cold-sensitive phenotype expressed by the mutated genomes, whether introduced into CREF cells by infection with virions or by transfection with intact genomes or plasmids, implies that the E1a 51-kd protein interacts with a viral (E1a 12S gene product) or a host macromolecule(s) or both. The cold-sensitive phenotype probably reflects a decreased binding constant at 32°C between the mutants' truncated proteins and a still unidentified macromolecule(s). Identification of the components involved and the characteristics of the viral protein macromolecular interactions should reveal critical clues to understanding the mechanisms of viral transformation.

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