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Transformation-Defective Mutant of Adenovirus Type 5 Containing a Single Altered E1a mRNA Species

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A mutant of adenovirus type 5 containing an octanucleotide insert in region E1a of the viral genome was constructed. The insert was present in only one (13*s*) of the three overlapping mRNA's synthesized from this region. The insert was within the sequences removed by RNA splicing during the production of the other two mRNA's. The insertion resulted in a shift in the translational reading frame of the 13*s* mRNA and the probable premature termination of translation. The mutant was defective for viral DNA replication in HeLa cells and the transformation of rat embryo and baby rat kidney cells, indicating that a product encoded by the 13*s* mRNA is required for these two processes. Other early regions of the genome were expressed in HeLa cells infected by this mutant although in some cases the expression was decreased as compared with wild-type-infected cells.

The transformation of cells by human adenoviruses requires the expression of the left-hand-end 11% of the viral genome. All adenovirus-transformed cell lines contain and express these sequences (7-9, 22-24). Studies involving the transfection of cells by specific fragments of the viral DNA have shown that the left terminal 8% of the genome contains the same amount of transforming activity as the intact genome (11, 16). All of the viral information necessary to initiate and maintain the transformed phenotype is thus contained within this region. The left-hand-end region (region E1) is one of several regions on the genome expressed at early times of infection before the onset of viral DNA replication (5). It is subdivided into two parts (E1a, 1.5 to 4.5 map units, and E1b, 4.5 to 11 map units), each of which encodes a family of overlapping mRNA's derived by the differential processing of common precursors (3-5, 20).

Mutants of adenovirus type 5 (Ad5) containing alterations in region E1 have been isolated and characterized. The mutants were all isolated and grown on 293 cells (Ad5-transformed human embryonic kidney cells [13]), which, by virtue of containing and expressing E1 DNA, complement mutants defective in this region. Jones and Shenk isolated a series of mutants that contained deletions of various extents which removed sequences from E1a and E1b (18, 25). An analysis of the transforming ability of these mutants indicated that both regions encoded products required for the productive growth of the virus and for the transformation of rat embryo cells (18). Harrison et al. have isolated and characterized a number of host range mutants which contain minor alterations (probably point mutations) located in E1a and E1b (15). Their results essentially agree with those obtained with the deletion mutants.

*dl*312, a mutant which contains a deletion removing most of early region E1a, was found to be defective for a number of viral functions; it was unable to transform rat embryo cells or to replicate its DNA and, in addition, failed to express other regions of the genome normally expressed at early times of infection (18, 19). Further analysis of this multifunctional E1a region is complicated by the fact that it encodes overlapping mRNA's. The deletion in *dl*312 alters all of these mRNA's, thereby making it impossible to assign any particular function to individual E1a products. Further investigations will require mutants containing alterations which differentially affect these products. We describe in this paper the isolation and characterization of a mutant containing an octanucleotide insert resulting in a single E1a mRNA species being altered.

MATERIALS AND METHODS

Cells and viruses. 293 and HeLa cells were propagated as described previously (17). Baby rat kidney cells were prepared from 6-day-old rats and propagated in Dulbecco modified essential medium containing 10% calf serum. Wild-type Ad5 (H5 300) was originally obtained from H. Ginsberg. Plaque assays on 293 and HeLa monolayers were performed as described previously (17). Infections with Ad5 DNA utilized the calcium phosphate precipitation method (14).

Restriction endonuclease digestions and gel

electrophoresis. DNA was prepared from purified virions as described previously (17). All enzymes were obtained from Bethesda Research Laboratories or New England Biolabs and used as directed. Restriction fragments were separated through 1% agarose gels and visualized by staining the gel with ethidium bromide.

Preparation of cytoplasmic RNA and S1 mapping. Cytoplasmic RNA was isolated from HeLa Spinner cells 8 h after infection as described previously (18). Cytosine arabinoside (25 μg/ml) was added to the cells 1 h after infection.

S1 mapping was carried out essentially as described by Berk and Sharp (2). ^{32}P-labeled DNA fragments (5-μg equivalents per ml) were hybridized to polyadenylic acid-containing RNA (500 μg/ml) in 20 μl of formamide at 59°C. The RNA-DNA hybrids were digested with S1 endonuclease (40 U in 200 μl of 30 mM NaOAc–250 mM NaCl–1 mM $ZnCl_2$–5% glycerol) for 30 min at 37°C. Resistant fragments were separated by electrophoresis in 3.8% polyacrylamide gels containing 8 M urea and Tris-borate buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA) and visualized by autoradiography.

DNA replication assay. HeLa or 293 cells (60-mm dish containing approximately 5×10^6 cells) were infected at a multiplicity of infection (MOI) of 50 PFU per cell and labeled with [^{3}H]thymidine (100 μCi per plate) 6 to 24 h after infection. DNA was prepared by digestion of the cells with 50 μg of proteinase K per ml in 10 mM Tris (pH 8.0)–1 mM EDTA–1% sodium dodecyl sulfate for 5 h at 37°C and then repeated extractions with phenol and chloroform-isoamyl alcohol (24:1). RNA was digested with RNase T_1 (1,000 U/ml) and RNase A (50 μg/ml) in 100 mM Tris (pH 8.0)–20 mM $MgCl_2$–0.4 M NaCl. The DNA was reextracted with phenol and chloroform-isoamyl alcohol, and approximately 2 μg of the labeled DNA was centrifuged to equilibrium in CsCl (1.7 g/cm^3 in 10 mM Tris [pH 8.0]–1 mM EDTA). Samples were collected, and their radioactivity was counted.

Transformation of baby rat kidney cells. Subconfluent cultures of primary rat kidney cells (approximately 10^6 cells per 60-mm dish) were infected with 0.2 ml of virus at various titers. After incubation at 37°C for 1 h, 5 ml of Dulbecco modified Eagle medium containing 10% calf serum was added. After 3 days the medium was changed to Spinner minimal essential medium containing 10% calf serum, which was replaced every 3 to 4 days. Transformed foci were counted after 4 weeks.

Construction of H5*in*500. A 200-μg sample of mutant H5 309 DNA (18) was digested with the restriction endonuclease *Sma*I. A phosphorylated linker molecule (0.01 optical density unit at 260 nm) of the sequence dGGAATTCC was added together with 5 U of T4 DNA ligase. After incubation at 4°C for 16 h, the ligated DNA was ethanol precipitated and digested with *Sma*I. Additional linker (0.005 optical density unit at 260 nm) was added, and the mixture was ligated at 4°C for 16 h. After another digestion with *Sma*I, more linker was added, and ligation was repeated one more time. This procedure was employed to maximize the percentage of *Sma*I fragments containing linker molecules ligated to their termini. After the final ligation, the DNA was restricted with *Eco*RI, and fragments migrating at the positions of *Sma*I-K (0 to 2.8 map units) and *Sma*I-F (2.8 to 11.0 map units) were isolated. The two fragments were ligated, and a fused F/K fragment was separated by gel electrophoresis and extracted. The fused fragment was digested with *Xba*I, the *Xba*I A fragment from mutant 309 DNA (3.8 to 100 map units) was added, and the mixture was ligated for 16 h at 16°C. The ligated DNA was used to transfect 293 cells in a DNA plaque assay. Individual plaques were picked and used to infect 293 cells. The resulting virus was purified; viral DNA was extracted and analyzed by restriction with *Sma*I and *Eco*RI. Of the resulting colonies, 33% had the H5*in*500 genotype.

RESULTS

Construction of H5*in*500. The three overlapping mRNA's encoded within region E1a are shown in Fig. 1. All three share the same 5′ and 3′ sequences, but differ in the size of internal sequences removed during RNA splicing. The 13*s* and 12*s* species predominate at early times of infection, and the smaller 9*s* species accumulates at later times (26). The *Sma*I restriction endonuclease cleavage site located at 2.8 map units (nucleotide position 1010) is within the coding sequences of the 13*s* mRNA, but within the intervening sequences of the 12*s* and 9*s* mRNA's (21). Alterations at this site, providing that they do not alter the synthesis and processing of RNA from this region, would result in the products encoded within the 13*s* mRNA being altered, but not those encoded by the other two mRNA species. The characterization of such a mutant would indicate the functional importance of the products of the 13*s* message.

An oligonucleotide of the sequence GGAATTCC was inserted into the *Sma*I cleavage site at 2.8 map units. The protocol used for accomplishing this is outlined in Fig. 2 and described in detail above. Mutant 309 DNA, which contains a single *Xba*I cleavage site at 3.8 map units (18), was restricted by *Sma*I. The octanucleotide linker molecule was added to the restricted DNA at high linker-to-DNA ratios, and the mixture was ligated with T4 DNA ligase. After restriction with *Eco*RI, the DNA was separated by agarose gel electrophoresis, and fragments migrating at the positions of *Sma*I-F and *Sma*I-K were isolated. Ligation of the linker molecule to the termini of the *Sma*I-generated fragments and then restriction with *Eco*RI would result in fragments containing *Eco*RI-generated cohesive termini (the linker molecule contains the recognition sequence for *Eco*RI). The resulting F and K fragments were ligated and subsequently restricted with *Xba*I (there is a single cleavage site within the F fragment located at 3.8 map units). A fragment extending from 3.8 to 100 map units generated by restrict-

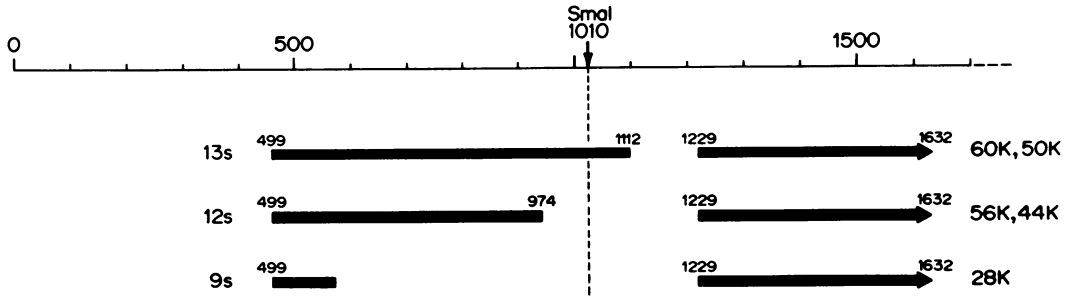

FIG. 1. *Map of the mRNA species synthesized from region E1a. The data are taken from Chow et al. (4) and from Berk and Sharp (3). Although the data relate to Ad2, the Ad5 map appears to be identical (3). The lines represent coding sequences, arrowheads represent polyadenylated 3' ends, and the gaps represent intervening sequences which are removed. The nucleotide positions of the 5' and 3' ends and of the splice junctions of the 13s and 12s mRNA's are taken from Perricaudet et al. (21). The location of the SmaI restriction endonuclease cleavage site in relation to the mRNA's is indicated. The molecular weights (in thousands) of the polypeptides synthesized in vitro from each of the E1a messages are shown and taken from the work of Esche et al. (6).*

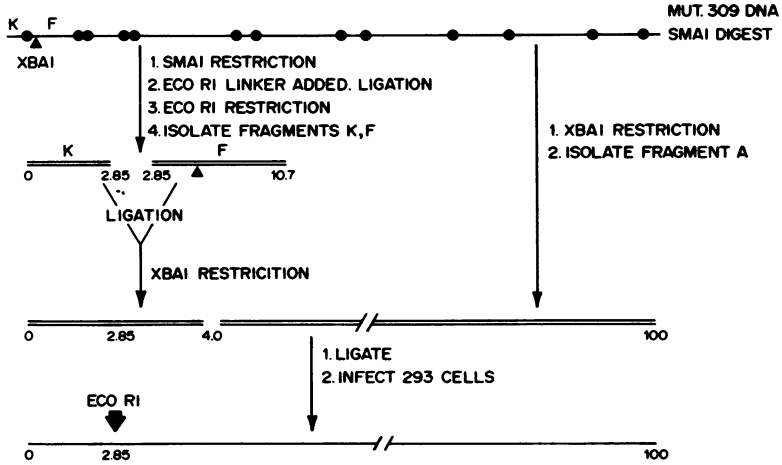

FIG. 2. *Protocol for the construction of H5in500. Details are given in the text.*

ing mutant 309 DNA with *Xba*I was added; the mixture was religated and then used to infect 293 cells in a DNA plaque assay.

Viral DNA from the resulting plaques was screened for the presence of an *Eco*RI cleavage site at 2.8 map units. Mutant 309 DNA contains a single *Eco*RI cleavage site at 76 map units; thus, restriction of this DNA with *Eco*RI resulted in two fragments (Fig. 3a). A mutant was obtained (H5*in*500) that gave three fragments when restricted with *Eco*RI (Fig. 3b). H5*in*500 DNA therefore contains an extra *Eco*RI cleavage site. Restriction of this DNA with *Sma*I resulted in a fused F/K fragment, showing that the site at 2.8 map units had been lost (Fig. 3d). Further restriction with *Eco*RI generated fragments migrating at the positions of *Sma*I-K and *Sma*I-F, demonstrating that the loss of the *Sma*I site at 2.8 map units was accompanied by the gain of an *Eco*RI site at this same position (Fig. 3f). The presence of the complete octanucleotide insert in the middle of the *Sma*I cleavage site at 2.8 map units was confirmed by direct sequence analysis (data not shown).

Host range phenotype of H5*in*500. The ability of H5*in*500 to form plaques on HeLa and 293 cells was tested. Whereas wild-type Ad5 plaques with equal efficiencies on both cell types, H5*in*500 gave only 1.3×10^6 PFU/ml on HeLa cells, as compared with 1.75×10^9 PFU/ml on 293 cells. The plaque-forming efficiency of H5*in*500 on HeLa cells is therefore about 1,000-fold lower than on 293 cells, indicating that the alteration in this mutant affects the product or products required for productive growth of the virus. Other mutants with alterations in region E1a exhibit a similar host range phenotype (15, 18).

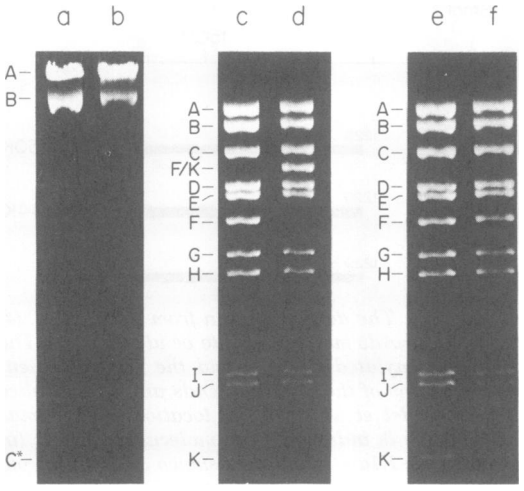

FIG. 3. *Electrophoretic analysis of fragments produced by cleavage of mutant 309 and H5in500 DNAs with EcoRI and SmaI. In each case 1 μg of DNA was cut, and the fragments were separated by electrophoresis on 1% agarose gels. (a and b) 309 and H5in500 DNAs cut with EcoRI. C* indicates the additional small fragment produced with H5in500. (c and d) 309 and H5in500 DNAs cut with SmaI. A fused F/K fragment is produced with H5in500 due to the loss of an SmaI cleavage site at 2.8 map units. (e and f) 309 and H5in500 DNAs cut with SmaI and EcoRI. Fragments migrating at the positions of SmaI-F and SmaI-K are produced with H5in500.*

Defectiveness of H5*in*500 for viral DNA replication. The analysis of other mutants with alterations in region E1a indicates that a product encoded within this region is required directly or indirectly for the replication of viral DNA (15, 18). Consequently, H5*in*500 was tested for its ability to synthesize viral DNA by labeling infected cells with [^{3}H]thymidine 6 to 24 h after the onset of infection (Fig. 4). DNA was extracted and centrifuged to equilibrium in CsCl. DNA prepared from 293 cells infected at an MOI of 50 PFU per cell predominantly sedimented at a position characteristic of Ad5 DNA (1.715 g/cm^3). DNA prepared from HeLa cells infected at the same MOI contained no detectable DNA at this position; the labeled DNA sedimented at a position characteristic of cellular DNA. Thus, the alteration in H5*in*500 affects a gene product which is required for the replication of viral DNA.

Transformation defectiveness of H5*in*500. The ability of H5*in*500 to transform rat embryo cells was tested. The assay used has been described previously (18). No foci of transformed rat embryo cells have ever been observed as a result of infection with H5*in*500 (data not shown). We conclude that a product altered in H5*in*500 is required for the transformation of rat embryo cells. Other mutants with alterations in region E1a have been found to be transformation defective (12, 18) when tested on rat embryo cells. H5*in*500 was also found to be deficient for the transformation of baby rat kidney cells. When very high MOIs were used, some transformants were obtained (Table 1). However, the frequency of such events was greatly decreased as compared with the frequency of transformation of these cells by wild-type Ad5. Maximum efficiencies of transformation with wild-type virus were obtained at low MOIs (less than 1 PFU per cell; Table 1), in accordance with other published findings (12). No transformed colonies

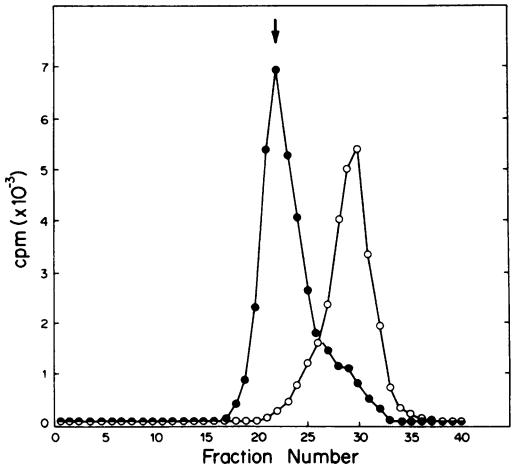

FIG. 4. *Viral DNA synthesis in H5in500-infected cells. HeLa and 293 cells were infected with H5in500 at an MOI of 50 PFU per cell, and the cells were labeled with [^{3}H]thymidine 6 to 24 h after infection as described in the text. DNA was extracted from each and separated by equilibrium centrifugation in CsCl, and the gradients were then fractionated. The radioactivity in each fraction is shown (●, 293 cells; ○, HeLa cells). The position where purified Ad5 DNA sediments in a similar gradient is indicated by the arrow.*

TABLE 1. *Transformation of baby rat kidney cells by wild-type Ad5 and H5in500*

Virus	MOI (PFU/cell)[a]	Total no. of transformed colonies[b]	Transformed colonies/PFU
Wild-type Ad5 (H5 300)	5	5	0.2/10^6
	0.5	5	5/10^6
	0.005	12	120/10^6
H5*in*500	50	3	0.03/10^6
	5	0	
	0.5	0	

[a] Approximately 10^6 cells per 60-mm dish.
[b] Total colonies from five dishes.

were observed when the cells were infected with H5*in*500 at these low MOIs.

Expression of early genes in H5*in*500-infected cells. Cytoplasmic RNA isolated 8 h after the infection of HeLa cells with H5*in*500 or wild-type virus was analyzed by the S1 mapping procedure of Berk and Sharp (2). The *Hin*dIII G fragment (0 to 7.8 map units) isolated from ^{32}P-labeled H5*in*500 DNA was used as a probe for region E1 RNAs. Hybridization of wild-type RNA to this probe followed by S1 digestion gave resistant bands of 1,100, 560, 485, 375, and 100 nucleotides in length (Fig. 5; the 100-nucleotide fragment is not visible in Fig. 5, but is clearly seen in the original autoradiograph). In contrast, H5*in*500 early RNA gave resistant bands of 1,100, 660, 485, and 375 nucleotides in length. The lack of a 660-nucleotide band in the wild-type RNA can be explained by the presence of a small, single-stranded loop between the wild-type RNA and the DNA probe at the position of the octanucleotide insert. S1 nuclease recognizes this loop and cleaves the normal 660-nucleotide band into fragments of 560 and 100 nucleotides in length. The 1,100-nucleotide fragment (and a faint 600-nucleotide fragment that is sometimes seen) results from hybridization of region E1b RNA to the probe; the remaining bands result from hybridization of E1a mRNA to the probe. Thus, E1a and E1b mRNA's of normal size were detected in H5*in*500-infected cells; the presence of the octanucleotide insert had no effect on the processing of these RNAs.

Region E1b, like other early regions of the genome, is regulated by a product or products encoded within E1a (1, 19). However, as indicated by the presence of the 1,100-nucleotide-long band in Fig. 5, the expression of region E1b is not impaired by the mutation in H5*in*500. The expression of early regions E2, E3, and E4 is also controlled by an E1a gene product (1, 19). Transcripts from each of these regions were detected in H5*in*500-infected cells. Figure 6 shows the results of the S1 mapping of wild-type and H5*in*500 RNAs by using a labeled DNA probe (the *Hin*dIII A fragment, 50.1 to 72.8 map units) that would detect region E2 mRNA. An S1-resistant band 1,700 nucleotides in length was obtained with both RNAs. However, the level of this band was consistently lower (usually between two and fivefold) in H5*in*500 RNA as compared with wild-type RNA. A similar analysis of regions E3 and E4 showed that RNA from region E3 was present at near wild-type levels in H5*in*500-infected cells, but that RNA from region E4 was present at levels significantly lower than was found in wild-type-infected cells (data not shown).

DISCUSSION

Early region E1a encodes products that demonstrate a number of pleiotropic effects. Inves-

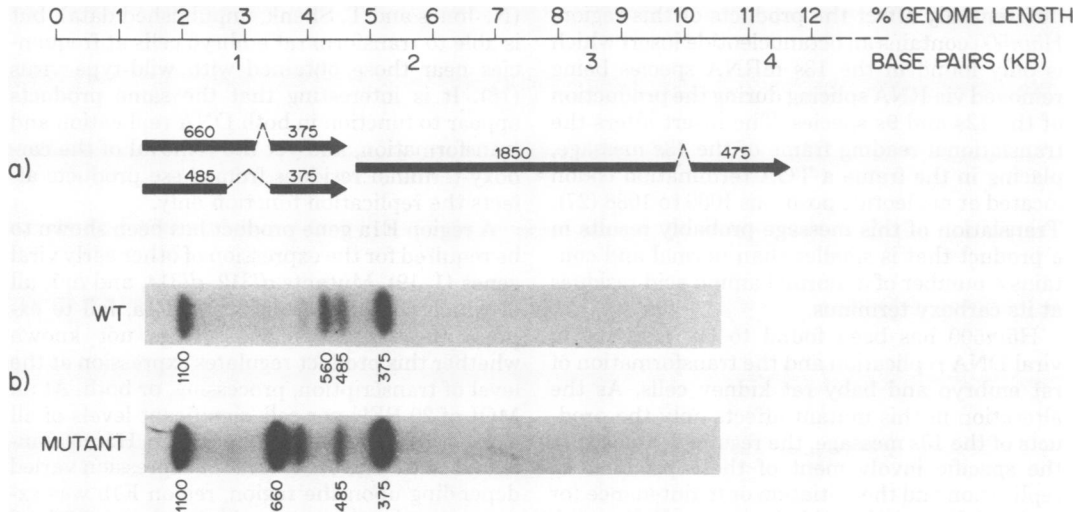

FIG. 5. *S1 mapping of region E1 RNAs. The structure and map locations of early RNAs synthesized from region E1 is shown (2). The autoradiograms represent S1-resistant DNA-RNA hybrids formed between the ^{32}P-labeled HindIII G fragment (0 to 7.8 map units) of the H5in500 genome and early cytoplasmic RNA (isolated 8 h after infection) from wild-type and H5in500-infected HeLa cells. In both cases the MOI was 20 PFU per cell. The hybrids were separated on a 3.8% polyacrylamide gel containing 8 M urea. The sizes of the fragments in nucleotides are indicated.*

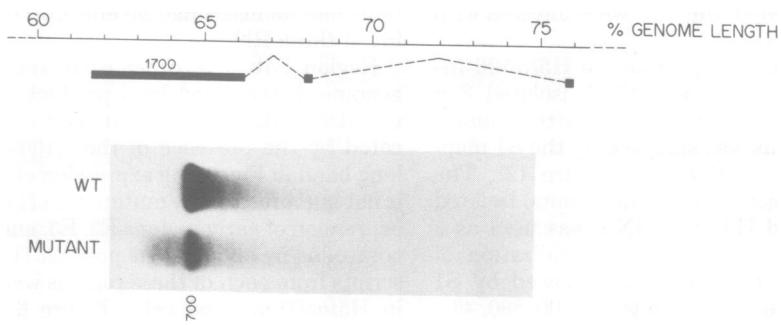

FIG. 6. *S1 mapping of region E2 RNAs. The cytoplasmic RNAs analyzed were the same as in Fig. 5. The ^{32}P-labeled probe was the HindIII A fragment (50.1 to 72.8 map units) isolated from H5in500 DNA. The S1-resistant hybrids were separated on a 3.8% polyacrylamide gel containing 8 M urea. The structure and map location of Ad2 region E2 RNA are taken from Berk and Sharp (3). WT, Wild type.*

tigations of mutants containing alterations in this region have shown that these products are required directly or indirectly for the transformation of rat embryo cells, for the replication of viral DNA, and for the expression of a number of other regions of the genome early after infection (1, 12, 15, 18, 19, 25). It is difficult to assign any one of these functions to particular products encoded within this region because overlapping mRNA's are produced (3–5, 20). In all previously characterized mutants containing a precisely known alteration in region E1a, all mRNA's are equally affected. To explore this region in more detail, we have directed our efforts toward isolating mutants that contain alterations which differentially affect the products of this region. H5*in*500 contains an octanucleotide insert which is only found in the 13*s* mRNA species being removed via RNA splicing during the production of the 12*s* and 9*s* species. The insert alters the translational reading frame of the 13*s* message, placing in the frame a TGA termination codon located at nucleotide positions 1086 to 1088 (27). Translation of this message probably results in a product that is smaller than normal and contains a number of abnormal amino acid residues at its carboxy terminus.

H5*in*500 has been found to be defective in viral DNA replication and the transformation of rat embryo and baby rat kidney cells. As the alteration in this mutant affects only the products of the 13*s* message, the results demonstrate the specific involvement of these products in replication and the initiation or maintenance (or both) of the transformed phenotype. Esche et al. have translated the 13*s* message in vitro; it was found to encode two polypeptides with molecular weights of 60,000 and 50,000 (6). It is not known why this apparently single mRNA species encodes more than one polypeptide although it is known that the source of the heterogeneity lies in the 3′ region of the message (6). At this point it cannot be ruled out that products encoded by the other E1a messages are required in addition to the 13*s* products for replication and transformation. Additional mutants that specifically alter the 12*s* or 9*s* product without affecting the 13*s* product would help answer this question. Mutant *dl*311 is a previously described mutant containing a deletion extending from 3.55 map units (nucleotide position 1281) to 3.72 map units (nucleotide position 1340 [18, 25]). As a result of the deletion, a single polypeptide is synthesized from the 13*s* message that lacks the normal carboxy-terminal amino acid residues (6). *dl*311 is defective for viral DNA replication (N. Jones and T. Shenk, unpublished data), but is able to transform rat embryo cells at frequencies near those obtained with wild-type virus (18). It is interesting that the same products appear to function in both DNA replication and transformation, and yet the removal of the carboxy-terminal residues from these products affects the replication function only.

A region E1a gene product has been shown to be required for the expression of other early viral genes (1, 19). Mutants *dl*312, *dl*314, and *hr*1, all of which contain alterations in E1a, fail to express these other regions. It is not known whether this product regulates expression at the level of transcription, processing, or both. At an MOI of 20 PFU per cell, significant levels of all early transcripts were detected in H5*in*500-infected cells. The exact level of expression varied depending upon the region; region E1b was expressed at levels comparable to those obtained during a wild-type infection, whereas region E2 was expressed at somewhat lower levels (usually two- to fivefold). Therefore, although the mutation in H5*in*500 does have some effect on the expression of other early viral genes, it is small compared with its effect on DNA replication and

transformation. It is possible that at lower MOIs the effect of the mutation on early gene expression would be more dramatic; this appears to be the case with mutant *hr*1 (1). However, we have had great difficulty in S1 mapping transcripts from H5*in*500-infected cells when MOIs of 5 or less have been used. This may indicate that at these low MOIs all messages, including those of region E1a, are decreased compared with the levels found in wild-type-infected cells. The exact role of the 13*s* products in gene expression is not clear. It is possible that the products encoded within the 12*s* and the 13*s* transcripts work in conjunction with each other to effect the efficient expression of early genes. The 12*s* products alone could be sufficiently active to allow the expression of all early genes, particularly at high MOIs. Alternatively, the 13*s* products may act alone in regulating early gene expression; in H5*in*500, although these products are altered, they could retain sufficient activity to effect normal expression of region E1b and somewhat decreased expression of region E2. We believe that the latter alternative is true. We have isolated and characterized several other mutants with alterations in region E1a which do not produce any detectable 12*s* products, but which do synthesize a product that is likely to be similar to the altered 13*s* product thought to be synthesized by H5*in*500 (unpublished data). The pattern of early gene expression in these mutants is similar to that of H5*in*500, suggesting that the 12*s* products play no major role in regulating this expression. With H5*in*500, at MOIs of 10 to 40 PFU per cell, DNA replication and transformation are clearly defective, but the expression of all early genes is normal or nearly normal; this suggests that the 13*s* products play a direct role in these two processes, rather than an indirect role via the regulation of other genes whose products are required.

*hr*1 is a mutant that contains an alteration (probably a point mutation) that has been genetically mapped to region E1a (10). In vitro translation studies have shown that the mutation results in products encoded within the 13*s* message being shortened with no apparent effect on the products of the 12*s* message (6). On the basis of these data, it is suggested that the *hr*1 mutation results in a nonsense codon somewhere in the region between 2.8 and 3.5 map units, i.e., the region included in the 13*s*, but not the 12*s*, message. As this is the region that contains the octanucleotide insert in H5*in*500, it might be expected that the two mutants would be phenotypically similar. Indeed, both were found to be defective for viral replication and the transformation of rat embryo cells. However, unlike H5*in*500, *hr*1 is able to transform baby rat kidney cells although the transformants obtained are abortive (12). A direct comparison of the mutants cannot be made until the exact lesion in *hr*1 is known. However, the difference in transformation properties of the two could suggest that the mutational site in *hr1* is to the right of nucleotide position 1010, which is the site of the octanucleotide insert in H5*in*500. The defective product of *hr*1 would in this case contain a larger portion of the normal, wild-type amino acid sequence and could as a result be more active in transformation than the defective product of H5*in*500.

ACKNOWLEDGMENTS

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