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Method for introducing site-specific mutations into adenovirus 2 genome: Construction of a small deletion mutant in VA-RNA$_I$ gene

(recombinant DNA/site-directed mutagenesis/DNA transfection/marker transfer)

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ABSTRACT **We have developed a method for introducing mutations into the adenovirus type 2 genome at predetermined sites. Specific mutations are introduced into segments of the viral genome cloned in bacteria by using plasmid vectors. The chimeric DNA is used to construct viral mutants by cotransfection with two viral DNA segments derived from both ends of the viral genome, each of which has overlapping sequence homology with the cloned viral DNA segment. To illustrate this procedure, we cloned a large restriction fragment [*Eco*RI fragment A, map position (mp) 0–58.5] by using plasmid vector pBR322, and a small deletion mutation was introduced at the *Bam*HI cleavage site located within the VA-RNA$_I$ gene (mp 29 at residue +75 on VA-RNA$_I$ gene). The mutated DNA was then used to construct viral mutants by cotransfection into human cells with the adenovirus type 2 DNA–protein complex digested with *Sal* I. *In vivo* recombination occurred via overlapping sequences between the cloned *Eco*RI fragment A and *Sal* I-digested DNA–protein complex at two sites, mp 0–25 (i.e., within *Sal* I fragment B) and mp 45–58.5 (i.e., overlapping sequences between *Sal* I fragment A and *Eco*RI fragment A), generating infectious DNA molecules with intact ends. The viral mutant grows as well as the wild type in KB cells and induces the synthesis of smaller VA-RNA$_I$ but normal-size VA-RNA$_{II}$.**

Adenoviruses (Ads) are excellent models for study of regulation of gene expression in animal cells. The viral genome consists of a linear double-stranded DNA molecule of approximately 35,000 base pairs (bp) (1). The regulation of expression of Ad genes and their role in cellular transformation have been studied to a limited extent by the use of various viral mutants. Several conditional lethal temperature-sensitive (*ts*) and host-range (*hr*) mutants have been isolated by mutagenesis of virus particles followed by screening for the desired phenotype (2, 3). Deletion, insertion, and substitution mutants of Ad have also been isolated by using biochemical methods designed to select viral mutants that preexisted in a virus stock (4–6).

Elegant work has been done with simian virus 40 by constructing deletion and point mutants by *in vitro* manipulation of the viral genome (7–11). Similar attempts with Ad are severely limited because the viral genome is linear and not amenable to *in vitro* manipulation as in the case of the simian virus 40 system. We have developed a method by which specific mutations at predetermined sites can be introduced into the viral genome cloned in bacteria by using plasmid vectors. The mutated chimeric plasmid DNA is then used to construct viral mutants by cotransfection with two viral DNA fragments from the ends of the viral genome that have overlapping sequence homology with the cloned, mutated DNA segment. Recombination between the mutated viral DNA in the plasmid and the cotransfected viral DNA fragments occurs *in vivo* to yield infectious viral genomic DNA. This method described here should be useful in constructing various viable and conditional lethal mutants such as host range and temperature-sensitive mutants of Ad at defined sites of the viral genome.

In this communication, we describe the construction of a small deletion mutant in the gene coding for the small VA-RNA$_I$. VA-RNA$_I$ is 157–160 nucleotides long and is made in large amounts during late stages of infection (12); it has been physically mapped on the viral genome at map position (mp) 29, spanning a *Bam*HI cleavage site (13, 14). Another related RNA, VA-RNA$_{II}$, whose synthesis begins at early stages, has been mapped to the right of VA-RNA$_I$ gene on the viral genome (15). Although considerable synthetic machinery of the virus-infected cells is used for the production of VA-RNAs, their role in virus multiplication remains obscure. Recently, it has been suggested that VA-RNAs play a role in processing viral transcripts (16, 17), analogous to that of the small cellular nuclear U-1 RNA (18, 19). Specific mutations in VA-RNA genes will be valuable in elucidating the role of VA-RNA in viral replication and gene regulation.

MATERIALS AND METHODS

Viral DNA, DNA–Protein Complex, DNA Transfection, and Isolation of Viral Mutants. Viral DNA was prepared as described (20). Ad2 DNA–protein complex was prepared by disrupting the virus particles with 4 M guanidinium·HCl, followed by chromatography on Sepharose 4B as described by Chinnadurai *et al.* (21). DNA transfections were carried out on 293 or KB cells by the calcium phosphate method of Graham and van der Eb (22). Individual plaques from the transfected dishes were isolated and screened for viral mutants (23).

Extraction and Gel Electrophoresis of RNA. Uninfected and infected cells were labeled with $H_3{}^{32}PO_4$ (100 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) in 60-mm dishes for 22 hr (2–24 hr after infection). Cells were collected and washed with phosphate-buffered saline, and the total cytoplasmic RNA was extracted as described (24). RNA was dissolved in 50 mM Tris/50 mM borate/8 M urea/0.05% bromophenol blue. Electrophoresis was carried out in 8 M urea/8% polyacrylamide gels (30 × 16 × 0.15 cm) at 400 V for 10 hr; autoradiography was on Kodak DF-85 x-ray film.

Plasmid Culture and DNA Isolation. Plasmid pBR322 (25) and host strain *Escherichia coli* HB101 were used in the cloning and mutagenesis experiments. Colonies of HB101 bearing plasmid pBR322 or derivatives were grown on L-broth plates supplemented with ampicillin (50 μg/ml). DNA preparations were made from liquid cultures propagated in M9 medium supplemented with thiamin (2 μg/ml) and 0.5% Casamino acids

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Abbreviations: Ad, adenovirus; bp, base pairs; mp, map position; WT, wild type; ExoIII, exonuclease III.

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(26). Chloramphemical (200 μg/ml) was added to cultures when the growth reached A_{590} nm = 1.0. Twelve to 16 hr later, the plasmid DNA was extracted from amplified cultures by treatment of the cells with hypotonic sucrose, lysozyme, and Triton X-100 (27). Plasmid DNA was banded in two successive CsCl/ethidium bromide gradients. For screening plasmid mutants, DNA was prepared from 30-ml cultures by using a scaled-down version of the above method but with only one banding of DNA in CsCl/ethidium bromide.

Construction of Recombinant DNA Molecules and Transformation of HB101. For construction of recombinant DNAs, the DNAs were digested with *Eco*RI (Miles) and *Bam*HI (Bethesda Research Laboratories, Rockville, MD). "Blunt" ends were generated by treating the DNA with S1 nuclease as described by Shenk *et al.* (28). Ligation reactions were carried out with T4 ligase (0.1–1.0 unit/ml) at 12°C overnight. Digestion of DNA with exonuclease III (ExoIII) was carried out in 70 mM Tris·HCl, pH 7.9/2 mM $MgCl_2$/1 mM 2-mercaptoethanol at 1.5 units/μg of DNA.

HB101 cells for transformation were harvested at A_{590nm} = 0.4–0.5, washed, and then treated with 30 mM $CaCl_2$ prior to the addition of DNA. After 2 hr at 0°C, the mixtures were held at 41°C for 30 sec, diluted with 10 vol of L broth, and incubated 1–2 hr at 37°C before spreading on ampicillin-containing L plates (29).

RESULTS

Cloning of *Eco*RI Fragment A (mp 0–58.5) of Ad2 DNA. In the work described here, we used an Ad2 DNA fragment from the left half of the viral genome (mp 0–58.5) generated by *Eco*RI. Ad2 DNA has blocked 5′ ends (30), which make it inaccessible for the ligation reaction. We therefore removed about 50 bp from the termini of the duplex DNA by sequential digestion with ExoIII and S1 nuclease. This DNA was cleaved with *Eco*RI. EcoRI fragment A was purified from agarose gels and used for construction of recombinant DNA. Plasmid pBR322 DNA was cleaved with *Bam*HI, and blunt ends were generated at the *Bam*HI cleavage site by digestion with S1 nuclease. The *Bam*HI site of the vector was removed to avoid an additional *Bam*HI site in the chimeric DNA because the mutations were subsequently generated at one of the two *Bam*HI sites on the viral DNA. The plasmid DNA was subsequently digested with *Eco*RI. The larger fragment having the intact gene coding for ampicillin resistance and origin of DNA replication was purified from agarose gels. The plasmid DNA and viral DNA were ligated and used for transformation of HB101 to ampicillin resistance. Ten colonies were picked, and plasmid DNA was prepared and analyzed for the presence of *Eco*RI fragment A by digestion with *Hin*dIII. The *Hin*dIII digestion pattern of one of the plasmids (pKC 301) is shown in Fig. 1. The chimeric plasmid produced fragments C, B, I, J, and D and a fusion fragment derived from the left and right ends of *Eco*RI fragment A and the vector DNA, as expected. All 10 colonies examined had identical structures.

Use of Cloned *Eco*RI Fragment A in the Reconstruction of Infectious Genomic DNA. We expected that, when cotransfected into human cells together with two DNA fragments derived from the two termini of the viral genome which have overlapping sequence homology with *Eco*RI fragment A, the cloned *Eco*RI fragment A would generate infectious genomic DNA by *in vivo* recombination, as shown previously (23). We therefore limit-digested Ad2 terminal DNA protein complex (31) with *Sal* I which cuts Ad2 DNA three times. *Sal* I fragment A (mp 45.9–100) and fragment B (mp 0–26) have overlapping sequence

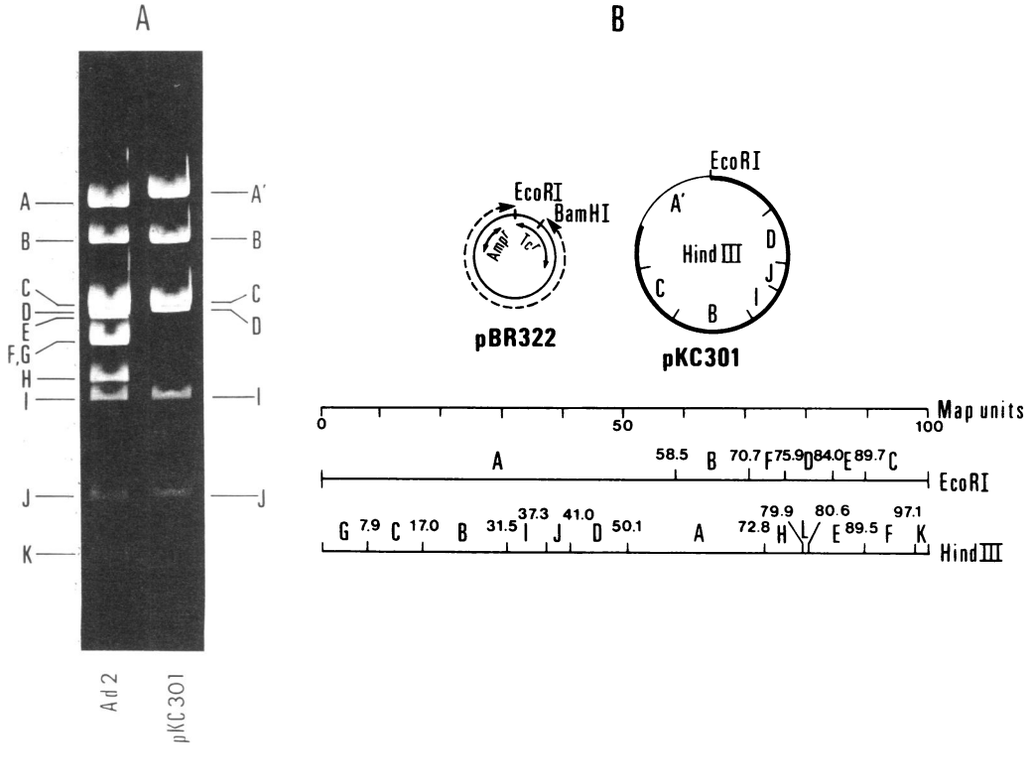

FIG. 1. Restriction cleavage patterns and cleavage maps of Ad2 and plasmid DNAs. (*A*) *Hin*dIII cleavage patterns of Ad2 and pKC301 DNAs. The DNA fragments were fractionated in 1% agarose gels, stained with ethidium bromide, and photographed. (*B*) Restriction maps of pBR322 and pKC301 DNAs. The dashed line indicates the fragment of pBR322 DNA used for cloning Ad2 *Eco*RI fragment A. The thick line in the pKC301 map indicates the viral DNA. C, B, I, J, and D represent the Ad2 *Hin*dIII fragments; A′ represents the fusion fragment of vector and viral DNAs. The *Eco*RI and *Hin*dIII cleavage maps (on an expanded scale) of Ad2 genomic DNA are shown below. The numbers indicate the mp of the restriction sites on Ad2 DNA.

Table 1. Rescue of transfection infectivity of *Sal* I-digested DNA–protein complex of Ad2 by pKC301 DNA

Dish	Plaques/dish		
	DNA–protein complex	pKC301	DNA–protein complex + pKC301
1	1	0	15
2	3	0	8
3	0	0	9
4	1	0	11

Transfection was carried out in 293 cells. *Sal* I-digested DNA–protein complex was used at 1 μg per dish and pKC301 DNA was used at 9μg per dish.

homology with *Eco*RI fragment A. *Sal* I-digested DNA–protein complex had very low infectivity when transfected into 293 or KB cells, probably due to some residual undigested genomic DNA. However, when cotransfected with pKC301 DNA, about 10-fold more plaques were produced (Table 1). This indicates that pKC301 DNA rescued some of the infectivity of the *Sal* I-digested DNA protein complex, presumably by a double recombination event between pKC301 DNA and *Sal* I fragments A and B, as illustrated in Fig. 2.

We did not remove *Sal* I fragment C from the DNA–protein complex mixture of *Sal* I fragments because it would not interfere with the generation of the desired recombinant—i.e., incorporation of *Sal* I fragment C into the recombinant needs two additional recombination events, and the frequency with which such multiple recombination occurs will be very low and may not be detected in our experiments. In generating infectious recombinant DNA, the two recombination events must have occurred somewhere between mp 0–26 (within *Sal* I fragment B) and mp 45.9–58.5 (overlapping sequences) between *Sal* I fragment A and *Eco*RI fragment A (pKC301). (Viable recombinants presumably will not form by a single recombination between the cloned *Eco*RI fragment A and *Sal* I fragment A because the terminal sequence was removed from *Eco*RI fragment A by ExoIII/S1 nuclease digestion.)

The interpretation that infectious genomic DNA is generated by a double recombination event is greatly strengthened by the fact that a deletion marker that was present at mp 3.8 in the Ad2 sequences in pKC301 was not present in the viral genomes obtained from these cotransfection experiments (unpublished data) when *Sal* I-digested DNA–protein complex was used in the cotransfection. That is, the left portion of the viable genomes must have come from *Sal* I fragment B of the DNA–protein complex. This provides the possibility of using the above transfection system to introduce mutations into the viral genome from mp 26 to mp 46 via the cloned viral DNA fragment.

Construction of a Small Deletion Mutant in VA-RNA$_I$ Gene. Using the above transfection system, we introduced a small deletion mutation into Ad2 genome at a *Bam*HI cleavage site located at mp 29.0 which lies within VA-RNA$_I$ gene at residue +75 (13, 14). pKC301 DNA which has two *Bam*HI sites at mp 29 and mp 42 (of the viral DNA) was linearized by partial digestion with *Bam*HI. The linearized plasmid DNA was treated with S1 nuclease to remove the cohesive ends generated by *Bam*HI. The blunt end molecules were circularized with T4 ligase at low DNA concentrations (<1 μg/ml) and used to transform HB101. DNA from individual colonies were screened for the absence of the *Bam*HI site by cleaving the plasmid DNA with *Bam*HI and *Kpn* I (results not shown). From the known cleavage maps of Ad2 DNA (32), the plasmids lacking the *Bam*HI site at mp 29 were identified. One of the plasmids, pKC311, was used for construction of viral mutants. Circular pKC311 DNA was cotransfected with DNA protein complex from Ad2 wild type (WT) digested with *Sal* I, and plaques were allowed to form on 293 and KB cells. Several plaques were picked at random and 10 were screened by cleaving the viral DNA with *Bam*HI. Nine of the 10 isolates lacked the *Bam*HI site at mp 29. The *Bam*HI cleavage patterns of Ad2 WT and one of the mutants, *dl* 401, are shown in Fig. 3. The deletion mutant produced normal fragment A (mp 59.5–100) and fragment C (mp 42–59.5); however, fragment B (mp 0–29) and fragment D (mp 29–42) were fused together in the mutant and migrated slightly slower (A′) than fragment A.

VA-RNA$_I$ Synthesis in Mutant-Infected Cells. To examine the effect of the deletion in *dl* 401 on the synthesis of VA-RNAs,

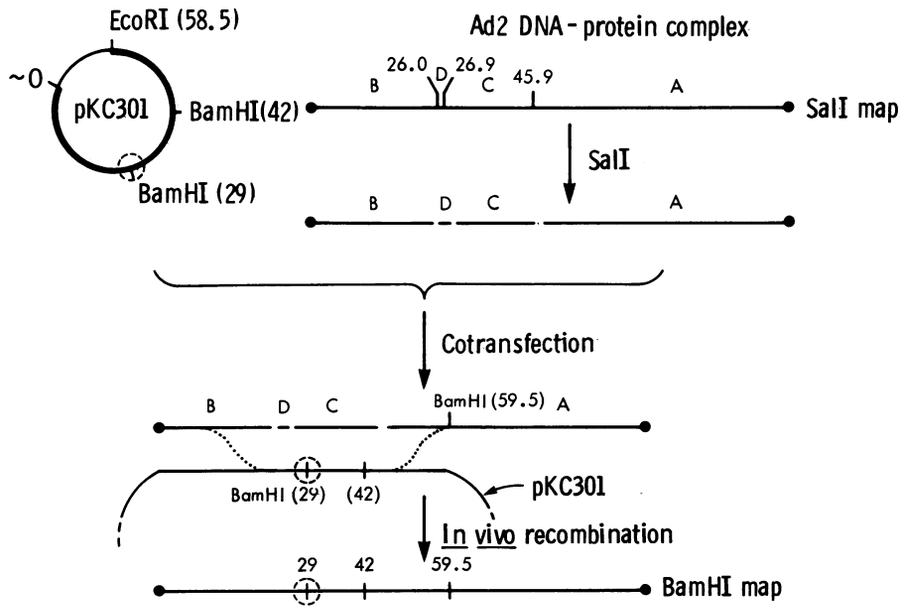

FIG. 2. Scheme for reconstruction of infectious genomic Ad2 DNA. The viral DNA is shown as thick lines. The numbers indicate the mp of the restriction sites. The dashed circle indicates the *Bam*HI site at which deletion mutations were constructed in subsequent experiments.

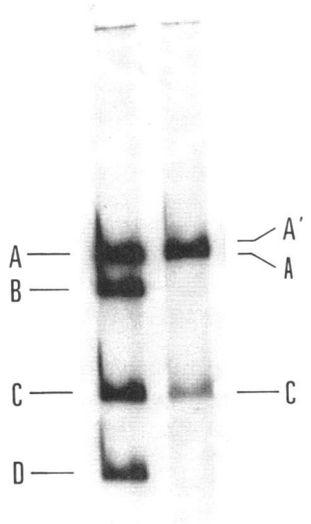

FIG. 3. Autoradiogram of Ad2 WT and *dl* 401 DNAs after cleavage with *Bam*HI. ^{32}P-Labeled DNA fragments were resolved in 1% agarose gels (20 × 20 × 0.3 cm), electrophoresed at 40 V for 16 hr, and autoradiographed. See Fig. 2 for *Bam*HI cleavage map of Ad2 DNA and the mutated *Bam*HI site in *dl* 401.

cells infected with Ad2 or *dl* 401 were labeled with ^{32}P, and the RNAs were extracted from the cytoplasm and analyzed by electrophoresis on 8% polyacrylamide gels containing 8 M urea. Mutant *dl* 401 synthesized two forms of VA-RNA$_I$ [VA-RNA$_I$ (A) and VA-RNA$_I$(G)] and VA-RNA$_{II}$ (Fig. 4). As expected, the VA-RNA$_I$ had slightly reduced size (<10 nucleotides). VA-RNA$_I$(A) is three nucleotides longer than VA-RNA$_I$(G) at the 5′ end (33), and both forms of VA-RNA$_I$ had correspondingly reduced sizes. This is expected because the deletion was introduced within the body of both forms of VA-RNA$_I$. The synthesis of VA-RNA$_{II}$, which has a separate promoter (15, 34) and maps to the right of VA-RNA$_I$ with a spacer of 98 nucleotides (35), was not affected by the deletion.

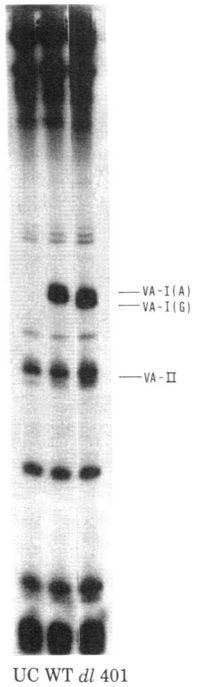

FIG. 4. Autoradiogram of low molecular weight RNAs from uninfected cells and cells infected with Ad2 WT or *dl* 401. UC, uninfected cells.

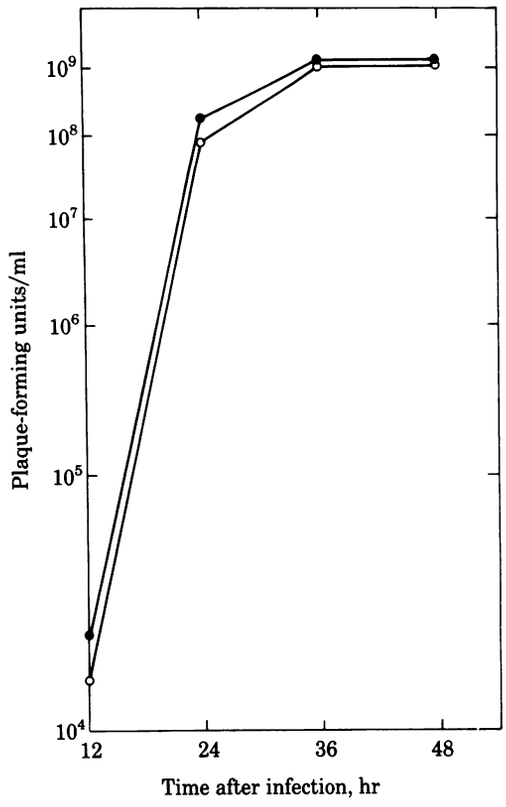

FIG. 5. Growth curve of Ad2 WT (●) and *dl* 401 (○). KB cells were infected with the WT or *dl* 401 at 5 plaque-forming units per cell in 60-mm dishes. After 1 hr for adsorption, the dishes were washed twice with phosphate-buffered saline and incubated with 5 ml of fresh medium. After various intervals, the infected dishes were frozen along with growth medium. Virus was liberated by sonication and titrated on KB cells.

Deletion Mutant in VA-RNA$_I$ Grows Normally. The growth properties of *dl* 401 were compared to those of Ad2 WT. KB cells were infected with WT or *dl* 401 at 5 plaque-forming units/cell, and the progeny virions were collected at various times after infection and plaque-assayed on KB cells. *dl* 401 grew as well as WT (Fig. 5).

DISCUSSION

We have developed a method for introducing specific mutations into Ad2 genome. In our method, mutations are introduced into segments of the viral genome cloned in bacteria by using plasmid vectors, and the amplified chimeric plasmid DNA is used to construct viral mutants by cotransfection of two viral DNA segments from both ends of the viral genome which have overlapping sequence homology with cloned viral DNA segment. This method is simple and yields little WT background. The small amount of WT background (1 of 10) observed in our present experiment was probably due to small amounts of undigested DNA–protein complex. In our subsequent experiments (data not shown), we could eliminate detectable WT background by extensive digestion of the DNA–protein complex with *Sal* I. This method should be applicable to other viruses with linear DNAs in which ends of the genome serve an essential role in viral replication.

An alternative approach to *in vivo* recombination is *in vitro* recombination of certain mutated cloned fragments [e.g., ligation of *Sal* I fragment C (mp 26.9–45.9) with Ad2 DNA–protein complex digested with *Sal* I]. It could be expected that the mutated cloned fragment will be incorporated in proper orientation in place of the WT fragment (4). This approach has several limitations. The cloned viral DNA fragment must be purified from the vector DNA. Moreover, *in vitro* ligation can be used only when the restriction endonuclease makes fewer cuts on the viral genome which is essential for constructing genomic DNA in proper orientation of the different fragments (4). This approach proved to be difficult in our hands. Using the method described here, we can introduce mutations anywhere between mp 25 and mp 90 of the Ad2 genome. We have successfully introduced several point mutations (8) at mp 36–88 by using appropriate cloned DNA fragments and suitable overlapping DNA fragments (unpublished data). At present we may not be able to introduce mutations at mp 0–25 of Ad2 genome for lack of suitable restriction endonucleases to make overlapping DNA fragments. However, the DNA–protein complexes from certain Ad variants that retain the restriction sites within the left 30% of the viral genome but have lost the sites on the right-hand side could be used successfully for this purpose. Such variants of Ad5 are already available (5). Our method should be useful in constructing various viable mutants such as regulatory mutants and conditional lethal temperature-sensitive and host range mutants. Cell lines that contain various integrated Ad genes are becoming available (36) and will be valuable in constructing specific host range mutants by the method described here.

Using the method described here, we have constructed small viable deletion mutants within VA-RNA$_I$ gene at residue +75. Recently, the sequences required for the initiation of transcription by RNA polymerase III have been observed to be intragenic (37). For VA-RNA$_I$ these sequences have been localized between positions +9 and +72 (38). Because *dl* 401 makes normal amounts of VA-RNA$_I$, the deletion must lie just outside the region required for initiation of transcription. The nucleotides deleted in the mutant are clearly nonessential for the functions of VA-RNA$_I$. Recently, a role for VA-RNAs in processing of viral transcripts analogous to the small cellular nuclear U-1 RNA (18, 19) has been suggested (16, 17). The VA-RNAs appear to have some sequence homology at the 5′ and 3′ ends to the 3′ and 5′ ends of viral mRNAs and have been shown to hybridize with these mRNAs (16, 17). If this is the sole function of VA-RNAs, viable deletions within this gene are possible as long as the secondary structures of the RNAs are not seriously affected. By using our method, it should be possible to construct further deletion mutants in VA-RNA$_I$ and VA-RNA$_{II}$ genes which will help us to understand the function(s) of these RNAs in viral development.

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