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Mutations in the Adenovirus Major Late Promoter: Effects on Viability and Transcription during Infection

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We developed an experimental system to examine the effects of mutations in the adenovirus major late promoter in its correct genomic location during a productive infection. A virus was constructed whose genome could be digested to give a rightward terminal DNA fragment extending from the *Xho*I site at 22.9 map units, which can be ligated or recombined with plasmid DNA containing adenovirus sequences extending from 0 to 22.9 or 26.5 map units, respectively. Mutations were made by bisulfite mutagenesis in the region between base pairs −52 and −12 with respect to the cap site at +1 and transferred to the appropriate plasmids for viral reconstruction. Of 19 mutant plasmid sequences containing single or multiple G-to-A transitions, 14 could be placed in the viral genome with no apparent change in phenotype. These mutant sequences included those which contained four transitions in the string of G residues immediately downstream of the TATA box. There were no alterations in rates of transcription from the major late promoter, sites of transcription initiation, or steady-state levels of late mRNAs. All of the five mutant sequences which could not be placed in virus contained multiple transitions both up- and downstream of the TATA box. Two of these apparently lethal mutant sequences were used in promoter fusion experiments to test their ability to promote transcription of rabbit β-globin sequences placed in the dispensable E1 region of the virus. Both sequences showed diminished ability compared with wild-type sequences to promote transcription in this context. Comparisons between these two sequences and the viable mutant sequences suggest a role for the string of G residues located between −38 and −33 in promoting transcription from the major late promoter. The data as a whole also demonstrate that the specific nucleotide sequence of this region of the major late promoter, which overlaps transcription elements of the divergent IVa_2 transcription unit and coding sequences of the adenovirus DNA polymerase, is not rigidly constrained but can mutate extensively without loss of these several functions.

The adenovirus major late promoter (MLP) has been studied extensively as a model for eucaryotic promoters. It contains a classic TATA box, necessary for correct and quantitative transcription in vitro (11, 22, 49), to which binds a transcription factor termed IID (41). As with many other eucaryotic promoters, a second sequence upstream from the cap site also affects the efficiency of transcription. This sequence element, centered at −58, is necessary for efficient transcription in vitro and in vivo (20, 23, 31) and is capable of binding a second transcription-promoting HeLa cell protein (7, 32, 41) termed USF.

In addition to these well-characterized upstream sequence elements, the MLP contains striking direct repeats of nonalternating GC pairs flanking the TATA box (see Table 1). It has been suggested, on theoretical grounds, that these repeats and similar sequences in cellular promoters may form a structure which acts as a "trap" for RNA polymerase II (6). A second model has proposed that the downstream G sequences may participate in the formation of a looped-out structure that is necessary for or enhances transcription initiation (55). While expression in vitro and in transfection is not affected significantly by transition mutations in these sequences (53, 54), the evolutionary conservation of the G stretches in all serotypes whose sequences have been determined (43a, 44) suggests an important structural or functional role during the normal replicative cycle.

The activity of the MLP is open to temporal regulation by *cis*- and *trans*-acting controls. During the early stage of an adenovirus infection, the MLP is transcribed at a low level (10, 42) and is dependent on *trans*-activation by products of the E1A region of the virus (38). After DNA replication, transcription from the MLP is apparently activated to higher levels in a *cis*-acting manner (45). Although E1A *trans*-activation of the MLP has been demonstrated in plasmid transfection experiments (26, 37), the temporal DNA replication-dependent regulation of this promoter has not been reconstituted in vitro or in transient transfection experiments with replicating plasmids (28).

Despite the increasing genetic and biochemical characterization of the MLP, no attempts have been made so far to determine the biological consequences of the various mutations examined. We have chosen therefore to develop an experimental system in which mutations in the MLP can be examined in their correct viral context. This decision was based also on the realization that the data gathered from experiments in vitro and transfection assays may not reveal the full interactive complexity of the controls operating on a particular promoter. Problems associated with inappropriate template topology, genomic position, and temporal and quantitative expression of *trans*-acting factors may obscure important elements of in vivo control. We also chose to focus on the G-rich regions flanking the TATA box in view of their possible role in defining the TATA signal.

Transition mutations were introduced into the G-rich regions by bisulfite mutagenesis. Mutant MLPs were tested for their ability to replace the wild type MLP (MLP-wt) by ligation or overlap recombination and then examined for

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transcriptional phenotype. Two of the five MLPs that were lethal in the construction assays were characterized further by being inserted upstream of a promoterless rabbit β-globin sequence placed in the adenovirus E1 region, in a manner similar to that used for other purposes by Logan and Shenk (27). This was imperative, as lethality could have arisen from changes in essential sequences on the opposite strand, in the coding sequence for DNA polymerase (2, 15), or the IVa_2 gene promoter (35, 36).

With these two reconstructive systems, it was shown that the MLP and overlapping DNA polymerase sequence are surprisingly amenable to change without phenotypic consequences. In particular, the G-rich sequence immediately downstream of the TATA box could be substantially modified without overt phenotypic effect. Sequence comparisons between the viable and nonviable mutant sequences suggest that the G-rich sequence upstream of the TATA box may be important in efficient transcription from the MLP.

MATERIALS AND METHODS

Transcription in isolated nuclei. Suspension cultures of HeLa or KB cells were infected at a multiplicity of 20 PFU/cell. At the appropriate times postinfection, nuclei were isolated (18, 21). Nuclei from 5×10^7 infected cells were incubated at 30°C for 15 min in a solution containing 1 mM GTP, CTP, and ATP, 1 μM UTP, and 200 μCi of [α-^{32}P]UTP (Amersham; 3,000 ci/mmol). Nuclei were disrupted by the addition of guanidinium isothiocyanate, and labeled nuclear RNA was purified by centrifugation through a cushion of CsCl (9). Labeled RNA was hydrolyzed by mild alkali cleavage and hybridized to a DNA dot matrix as described previously (12). Plasmid DNAs used for the hybridization contained various regions of adenovirus DNA: E1A (2.55 to 3.72 map units [m.u.]), L1 (32.15 to 37.94 m.u.), L3 (50.97 to 60.12 m.u.), and E4 (93.49 to 100 m.u.). (One map unit is equal to 359.37 base pairs [bp].)

To quantitate the hybridizations, radioactive regions were cut from the filters and counted by liquid scintillation, or autoradiograms were scanned densitometrically. Duplicate hybridizations containing half the labeled input RNA were performed at the same time to control for completion of hybridization.

Isolation of cytoplasmic RNA. After the nuclei were pelleted by centrifugation, the cytoplasmic supernatant was extracted twice with phenol-chloroform-isoamyl alcohol (24:24:1). Following ethanol precipitation, polyadenylated [poly(A)$^+$] RNA was isolated by oligo(dT)-cellulose chromatography (3).

Poly(A)$^+$ RNA was then subjected to Northern analysis (48); 0.2 μg of RNA from late time points was fractionated on a 1% agarose gel containing 2.2 M formaldehyde. After transfer of the RNA to nitrocellulose, the RNA was hybridized to plasmid DNA probes labeled by nick translation.

Primer extension analysis of late RNA from infected cells was performed as described (30) with a cloned hexon-specific 46-nucleotide primer (1). After hybridization of 5 μg of poly(A)$^+$ RNA to 4 pmol of the 5′-labeled primer, the primer was extended with avian myeloblastosis virus reverse transcriptase. The reaction products were separated on a 6% acrylamide sequencing gel containing 7 M urea, and the products were visualized by radioautography.

Construction of plasmids. The steps necessary to create plasmids with mutations in the MLP suitable for attempted reconstruction into viral genomes were as follows. The 300-bp fragment from the *Xho*I site at 16.1 m.u. to the *Pvu*II site at 16.9 m.u. (+33 with respect to the cap site) was subcloned after the addition of an *Eco*RI linker to the *Pvu*II end between the *Xho*I and *Eco*RI sites of pXho15, a derivative of pBR322 in which an *Xho*I linker has been substituted for bp 3608 to 4360 (24). The 41-bp fragment between the *Hpa*II (−52) and *Hha*I (−11) sites was removed and replaced with a *Sal*I linker. The resulting *Xho*I-*Eco*RI fragment containing the deletion was used to replace the homologous sequence in the adenovirus type 5 (Ad5) *Xho*I E fragment contained in pXhoIE-wt. The *Pvu*II site was lost during the construction and two extra base pairs of DNA were gained after S1 trimming of the linker and blunt end ligation in the formation of pXhoIE-dl.

Heteroduplex plasmids were formed with DNA from pXhoIE-wt and pXhoIE-dl (Fig. 1) as described (39). The DNA was treated with sodium bisulfite (43) for various times, and the modified DNA was transfected into *Escherichia coli* K58 Ung$^-$ (4). DNA from individual colonies was isolated, and nucleotide substitutions were identified by DNA sequence analysis (29). The altered *Xho*I E fragments were ligated into the *Xho*I site of pLB209 (5) or used to replace the *Xho*I E fragment from plasmid p0-26.5 (C. S. H. Young and P. I. Freimuth, unpublished data). It is important to note that both the wild-type and mutant forms of pXhoI-E, since they derive from the wild-type strand in the heteroduplex, contain the *Pvu*II site and lack the inserted *Sal*I site of pXhoIE-dl.

A clone of rabbit β-globin chromosomal DNA (46) was obtained from Carl Dobkin. The clone consists of a *Bgl*II partial digest fragment cloned into the *Bam*HI site of pBR322. The vector *Pvu*II site was converted to an *Xho*I site with linkers, leaving a single *Pvu*II site in the β-globin sequences immediately upstream of the cap site. This new plasmid, termed pβGX, was digested with *Xho*I and *Pvu*II, and the promoter-containing smaller fragment was replaced with the MLP-containing *Xho*I-*Pvu*II fragments from some of the pXhoI-E plasmids. These manipulations created a series of plasmids termed pAdβG. Transcription of the globin DNA sequences should be driven by the adenovirus MLP (Fig. 2).

For insertion into virus, the pAdβG series was modified further. The DNA was cut with *Xho*I, the ends were filled in with the Klenow fragment of *E. coli* DNA polymerase I, and *Bgl*II linkers were added (which restored the *Xho*I site). The ligated material was cleaved with *Bgl*II, and the fragment extending from the newly modified *Xho*I site to a *Bgl*II site internal to the β-globin sequences was cloned into pGem2Ad60.1. This latter plasmid contains the first 191 bp of Ad5 juxtaposed to a *Bgl*II site at bp 3322 followed by adenovirus sequences to the *Xho*I site at bp 5778 (16.1 m.u.). This in turn is followed by a polylinker. These manipulations created a series of plasmids termed pGemAdβG (Fig. 2), which all have the MLP and globin sequences directed toward the *Xho*I site at 16.1 m.u.

Construction of viruses. To create a suitable recipient for mutant MLPs, it was necessary first to create a virus whose genome would yield a right-hand DNA fragment extending to the *Xho*I site at 22.9 m.u. This was achieved as follows. P54 is an Ad5-Ad2$^+$ ND1 chimera containing *Xho*I sites at 16.1, 22.9, 26.9, and 68 m.u. (34). The 60.1 to 85 m.u. *Bam*HI fragment from P54 was inserted into the *Bam*HI site of pBR322 to create pAdBam68. Sodium bisulfite mutagenesis was performed on heteroduplexed DNA containing a single-stranded gap at the *Xho*I site introduced by treatment of one of the parental strands with *Xho*I and S1 nuclease. The other parental strand was obtained from pAdBam68 (linearized

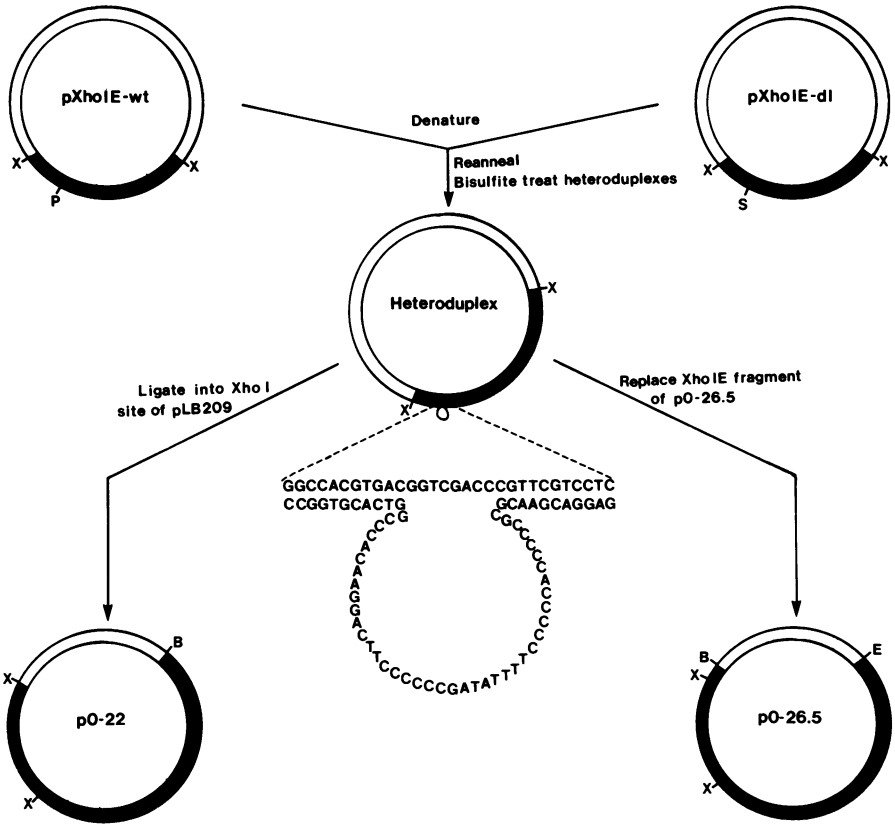

FIG. 1. Sodium bisulfite mutagenesis of a heteroduplexed plasmid formed from pXhoIE-wt and pXhoIE-dl. pXhoIE-wt plasmid DNA was linearized by digestion with *Bam*HI, and pXhoIE-dl plasmid DNA was linearized by digestion with *Pvu*II. The two sites are in the vector (not shown). The DNAs were mixed, denatured with alkali, and allowed to reanneal. The heteroduplexed plasmid DNA was treated with sodium bisulfite for 0.5 to 4 h. The treated DNAs were transfected into *E. coli* K58 cells, and tetracycline-resistant clones were screened for the presence of two *Pvu*II sites. The positive plasmids were sequenced to identify the mutations introduced by the treatment with sodium bisulfite. Only one of the two possible heteroduplexed plasmids is shown. X, *Xho*I; P, *Pvu*II; S, *Sal*I; B, *Bam*HI; E, *Eco*RI.

with *Sal*I). The gap was filled in with the Klenow fragment of *E. coli* DNA polymerase I. The altered *Bam*HI fragment was ligated to the terminal arms of $I_0$115.16 (33) produced by *Bam*HI digestion. After transfection of 293 cells and analysis of virus yields as described previously (47), the virus LL24 was identified. It lacked the *Xho*I site at 68 m.u. but had gained an additional Ad5-specific site at 28.6 m.u. derived from the left terminal arm of $I_0$115.16. This site was removed by overlap recombination. The LL24 DNA-protein complex was cleaved with *Sal*I, and the DNA-protein complex from strain 17 (34) was cleaved with *Eco*RI. The unpurified products were cotransfected into 293 cells, and virus yields were analyzed as before. LLX1 was isolated and the pattern of its viral DNA was examined by *Xho*I and *Pae*R7I digestion. The latter enzyme, an isoschizomer of *Xho*I, fails to cleave the *Xho*I site at 26.9 m.u. (14). Thus, LLX1 contains only two recognition sites for *Pae*R7I, at 16.1 and 22.9 m.u.

Replacement of the MLP in LLX1. Plasmid DNA from p0-22 containing a wild-type or mutant MLP was linearized with *Bam*HI and then partially digested with *Xho*I. The 0 to 22.9 m.u. fragment was isolated following separation in low-gelling-temperature agarose. LLX1 viral DNA was cleaved with *Pae*R7I, and the 22.9 to 100 m.u. fragment was isolated after two rounds of electrophoresis in low-gelling-temperature agarose. The fragments were quantitated by ethidium bromide staining and UV illumination. Equimolar amounts of the two fragments were ligated in the presence of 15% polyethylene glycol 6000 (40). After removal of the polyethylene glycol and ethanol precipitation, the DNA was transfected (13, 17) into 293 cells, virus was harvested, and virus titers were determined on HeLa cells for the isolation of single plaques.

Alternatively, p0-26.5 plasmid DNA containing either wild-type or mutant MLP was linearized at 26.5 m.u. by digestion with *Bam*HI and cotransfected into 293 cells with LLX1 DNA-protein complex digested with *Pae*R7I and *Cla*I. The latter cleaves at 2.6 m.u. in the chimeric Ad5-ND1 genome present in LLX1. Direct plaque assays were performed.

Construction of globin-containing viruses. DNA from pGemAdβG was cut with *Xba*I in the polylinker, yielding an adenovirus end at approximately 16.1 m.u. Several base pairs of the polylinker were attached to this end, but they did not prohibit overlap recombination.

DNA from H5*in*340 (19), a strain which contains the packaging signal at the right-hand end of the genome, was cleaved with *Xba*I and *Cla*I to yield a terminal fragment extending from 3.7 to 100 m.u. The two DNAs were transfected into 293 cells. Viral plaque populations were isolated directly, and the genome structures were determined by rapid analysis of low-molecular-weight DNA (47). Recombination between the plasmid and H5*in*340 DNAs in the 9.2 to 16.1 m.u. interval should generate the desired virus.

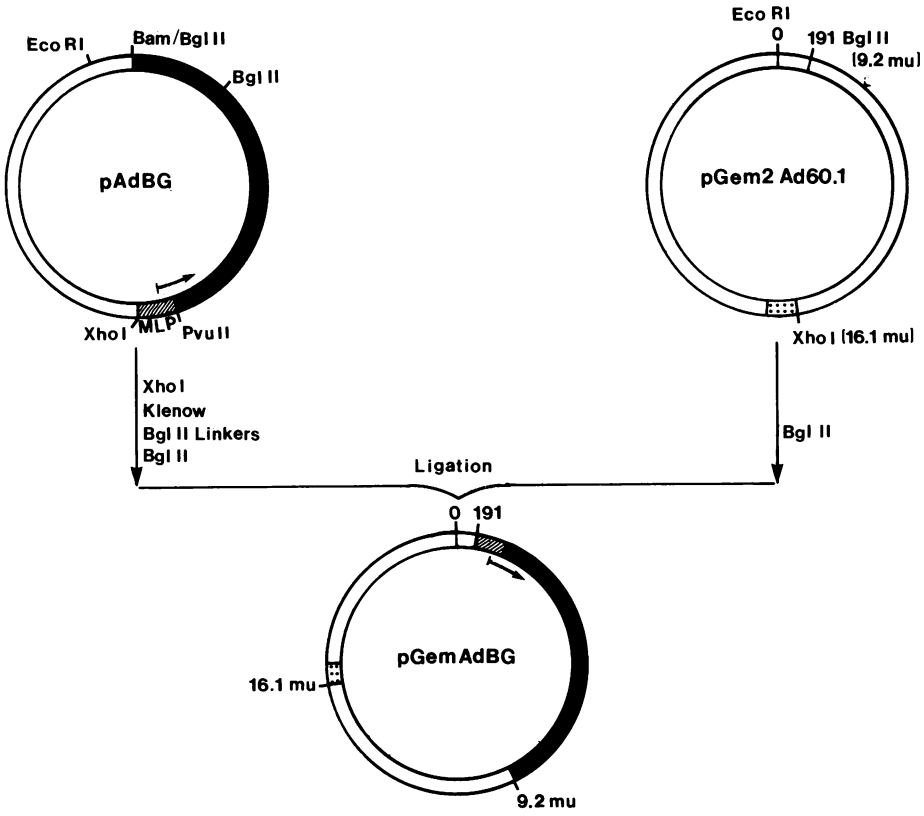

FIG. 2. Construction of the plasmids containing adenovirus MLP sequences and a promoterless rabbit β-globin sequence. The details of the construction of pAdβG and pGemAdβG are given in Materials and Methods. In pGemAdβG, the MLP sequences (hatched area) from the *Xho*I site at bp 5778 to the *Pvu*II site at bp 6069 are fused with the β-globin sequence (solid area), which in turn is followed by adenovirus sequences (open area) from the *Bgl*II site at bp 3322 to the *Xho*I site at bp 5778 (16.1 m.u.). This is followed by the gemini plasmid polylinker (stippled area) and then by plasmid sequences. The left-terminal 191 bp of adenovirus are also indicated.

RESULTS

Introduction of mutations into the adenovirus MLP in its correct genomic location. To create random transitions in the nonalternating GC repeats bracketing the TATA box of the MLP (see Table 1 for the wild-type sequence) and to avoid the introduction of potentially lethal deletions and insertions in the overlapping DNA polymerase gene, we chose to use sodium bisulfite mutagenesis (Fig. 1). Following mutagenesis, the mutant plasmid sequences were transferred to the appropriate plasmid backgrounds necessary for ligation or recombination with a viral right-hand genomic fragment from a newly produced virus termed LLX1 (see Materials and Methods for details). Figure 3 illustrates a ligation experiment using the viable sequence contained in MLP 9. Table 1 indicates the nucleotide sequences of all the altered MLPs obtained from the mutagenesis and whether particular mutations were viable. To establish firmly the lethality of the few mutations which were nonviable on a first transfection, the in vitro ligation, transfection, and plaque assay was repeated two more times with at least one different preparation of mutant plasmid DNA. In no case was viable mutant virus recovered, in contrast to control transfections performed in parallel with wild-type plasmid sequences, which always yielded virus.

Overlap recombination was used also to confirm the lethality of these mutations. Table 2 shows the results of four separate direct plaque assays, which demonstrated the failure to obtain virus with MLPs 13, 14, 17, and 18. The lower temperature of 32°C used in the first experiment (Table 2) was chosen in case potential amino acid changes in the DNA polymerase conferred temperature sensitivity on its function. From the results (Tables 1 and 2) it can be seen that no viable virus was obtained that contained mutant MLP 13, 14, 17, 18, or 19.

For the viable viruses, with the possible exception of MLP 12, no obvious phenotypic changes were observed. Plaque morphology, a known indicator of some adenovirus mutations (8), was normal for all mutants except MLP 12, which produced slightly smaller plaques. This phenotype was observed when a second plasmid was reconstructed from the viral MLP 12 *Xho*I-*Pvu*II fragment (−260 to +33) and reintroduced into virus by overlap recombination, limiting the basis of the phenotype to the MLP region and probably to the mutations present in the −52 to −11 sequence.

All of the viable viruses produced normal titers after growth of individual working stocks.

Transcriptional phenotype of some of the viable viruses. Although no gross new phenotypes were apparent for the recombinant viruses, it was possible that the transcription pattern from the MLP had been altered. Changes could exist in the overall rate of transcription, as measured indirectly by steady-state levels of late mRNAs or more directly by nuclear run-on transcription. Alternatively, the rate of transcription might not follow the usual temporal pattern of increase after the beginning of DNA replication. Finally, the site of initiation could be altered, so that the 5′ ends of mature RNAs were different. It was decided to look in more

TABLE 1. Changes in nucleotide sequence in the MLP region and putative amino acid changes in the DNA polymerase[a]

MLP	Nucleotide and amino acid changes
	790 780
	C-HisGlyProThrGlySerProProSerTyrPheProThrProAlaArg-N
w.t.	CGTGACCGGGTGTTCCTGAAGGGGGCTATAAAAGGGGGTGGGGGCGCG
	-50 -40 -30 -20 -10
1	Glu CGTGACCGGGTGTTTCTGAAGGGGGCTATAAAAGGGGGTGGGGGCGCG
2	Leu CGTGACCGGGTGTTCCTGAAGGAGGCTATAAAAGGGGGTGGGGGCGCG
3	Asn CGTGACCGGGTGTTCCTGAAGGGGGTTATAAAAGGGGGTGGGGGCGCG
4	CGTGACCGGGTGTTCCTGAAGGGGGCTATAAAAAGGGGTGGGGGCGCG
5	Thr CGTGACCGGGTGTTCCTGAAGGGGGCTATAAAAGGGGGTGGGGGTGCG
6	Asp Arg CGTGATCGGGTGTTCTTGAAGGGGGCTATAAAAGGGGGTGGGGGCGCG
7	Leu CGTGACCAAGTGTTCCTGAAGGGGGCTATAAAAGGGGGTGGGGGCGCG
8	Ile Ser CGTGACCGGGTATTCCTGAAGAGGGCTATAAAAGGGGGTGGGGGCGCG
9	Glu Asn CGTGACCGGGTGTTTCTGAAGGGGGTTATAAAAGGGGGTGGGGGCGCG
10	Lys CGTGACCGGGTGTTTTTGAAGGGGGCTATAAAAGGGGGTGGGGGCGCG
11	Ser CGTGACCGGATGTTCCTGAAGGGGGACTATAAAAAGGGGTAGGGGCGCG
12	Ile LeuLeu Phe CGTGACCGGGTATTCCTGAAAGGAGGCTATAAAAGAAGGTGGGGGCGCG
13	Ser LeuVal CGTGACCGGGTGTTCCTGAAGAAGGGCTATAAAAGGGGGTAAGGACGCG *
14	Leu Leu Ser CGTGACCGGGTGTTCCTGAAGGAAGGCTATAAAAGAGGGTGGAGGCACG *
15	Phe Ser CGTGACCGGGTGTTCCTGAAGGGGGCTATAAAAAAAAGTGGAGGCGCG
16	Ser Leu PheIlePhe CGTGACCGGATGTTCCTAAAGGGGGACTATAAAAGAAAATTGAAGGCGCG
17	Phe Phe Phe Val CGTGACCAAATGTTCCTGAAAAAGGACTATAAAAAAAGGTAGGGACACG *
18	Phe Phe SerIleSerVal CGTGACCAAATGTTCCTGAAAAAGGGCTATAAAAAGAAATGGAGACACG *
19	Phe LeuLeuSer PheIleSer CGTGACCGAATGTTCCTAAAAGGGAACTATAAAAGAAGATAGAAGCACG *

[a] The Ad2 MLP-wt sequence is shown from −58 to −10 (with respect to the cap site at +1); base pairs are marked beneath the sequence. The AT-rich TATA region is underlined in the wild-type sequence, and the nucleotide changes are underlined in the mutant sequences, with the predicted amino acid changes shown above. Amino acid data are from reference 2. Mutant MLPs which could not be placed in viable virus are indicated by asterisks.

detail at the transcription patterns exhibited by mutants with changes immediately adjacent to the TATA box (MLPs 4, 9, and 15) and the one mutant with diminished plaque size (MLP 12).

Steady-state levels of mRNA. Blot hybridization of cytoplasmic RNA from infected cells revealed no major differences in the steady-state level of mRNA from the MLPs at 18 h postinfection. MLP-12 and MLP-15 produced comparable levels of L1- and L3-specific mRNAs as MLP-wt (Fig. 4). MLP 12-infected cells appeared to contain more L1- and L3-specific RNA, but they also contained more actin RNA, a control for the amount of input RNA. Other RNA blot hybridization experiments did not reveal any consistent differences between MLP 12 and MLP 15 (data not shown).

Determination of transcription rates. Data from four separate run-on experiments with nuclei from cells infected with MLP-wt or MLP 4, 9, 12, or 15 are presented in Table 3. The levels of transcription at various late times were measured by using plasmid DNA sequences that could hybridize with transcripts from L1, L3, and E1A regions of the genome. Following normalization to the counts bound to the E1A filter, each mutant infection was compared with the wild-type infection in the same experiment. With most mutants, the relative level of transcription at all times was within 1.6-fold of the wild-type level. The exception, MLP 15, had only 36% of the wild-type level at 24 h in one experiment. However, in a second experiment, at the same time postinfection, the value was 1.3-fold that of the wild type. We suggest that the range of transcription rates in these experiments is within normal experimental variation, and no consistent pattern of an increase or decrease in the rate of transcription of these mutant MLPs in infected cells at late times could be detected. Similarly, no consistent differences in transcription rates from any of the promoters could be observed at 8 h postinfection (data not shown).

TABLE 2. Frequency of plaque formation after overlap recombination between p0-26.5 and LLX1[a]

MLP	No. of plaques			
	32°C	37°C	37°C	37°C (HeLa)
wt	119	41	24	9
13	0	0	0	0
14	0	0	0	0
17				0
18				0

[a] *Bam*HI-cleaved p0-26.5 plasmid DNA (200 ng) and 100 ng of *Pae*R7I-cleaved LLX1 DNA-protein complex were used per 60-mm dish of 293 cells or HeLa cells (last column) in a direct plaque assay. Three dishes were used per experiment, and the total number of plaques obtained on the three dishes is given. Each column represents one experiment. MLPs 17 and 18 were used in only one experiment.

5′ ends of late mRNAs. Since the TATA box is an important promoter element involved in positioning the RNA polymerase for correct initiation, the 5′ end of late mRNA from the MLP was determined for viruses containing mutations near the TATA box (MLPs 9, 12, and 15). The predicted 257-nucleotide fragment resulting from primer extension of hexon-specific mRNA (1) from cells infected with MLP-wt, 12, and 15 was present in each lane (Fig. 5). Similar results were obtained for MLP 9 (data not shown). No bands not present for MLP-wt were detected, nor was there any evidence of stuttering by the RNA polymerase during transcription initiation from the mutant MLPs. The smaller fragments generated during the reverse transcriptase elongation reaction were present in each lane and showed experimental variation (data not shown), suggesting pausing or premature termination by reverse transcriptase. Even though the TATA box was lengthened by four adenine residues (MLP 15), no improper initiation occurred, which is in agreement with the in vitro results of Yu and Manley (53). This suggests that the downstream nonalternating guanosine residues do not play a strong role in positioning the RNA polymerase for correct initiation.

Are MLPs 13 and 14 capable of promoting transcription? Since the mutant MLPs 13 and 14 were incapable of substituting for MLP-wt in virus, it was imperative to determine whether these promoters were capable of promoting transcription efficiently or whether other defects, e.g., mutations in the DNA polymerase, were responsible for the lethal phenotype. Transient expression of transfected MLP plas-

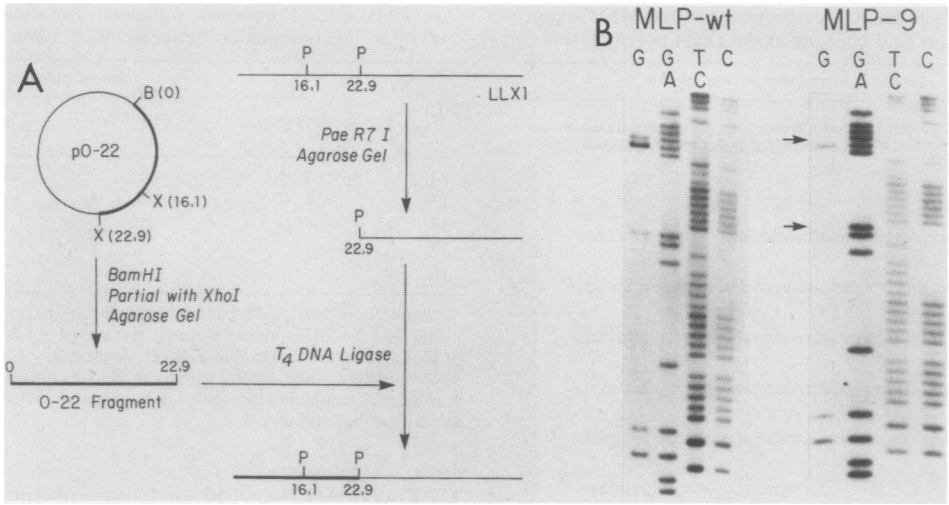

FIG. 3. Replacement of MLP-wt in LLX1 with an altered MLP. (A) p0-22 plasmid DNA was linearized by digestion with *Bam*HI (B) and then partially cleaved with *Xho*I (X). The purified 0 to 22.9 m.u. fragment was ligated to the purified 22.9 to 100 m.u. fragment produced by *Pae*R71 (P) digestion of LLX1 viral DNA. The ligation reactions were transfected into 293 cells, and virus yields were assayed for plaque formation on 293 cells. Individual plaques were isolated, and viral DNA was prepared. The *Xho*I E fragments were cloned into pMK2004, and the area surrounding the MLP of each fragment was sequenced. (B) Autoradiograph of an 8% sequencing gel of MLP-wt and MLP 9. The arrows indicate the locations of the nucleotide substitutions in MLP 9.

mids has proved to be an inefficient experimental method because of the low basal activity of the wild-type MLP in the absence of an enhancer or *trans*-activator (26, 37, 51). However, in experiments performed with a chloramphenicol acetyltransferase (CAT) plasmid assay, mutant MLP 14 consistently had lower CAT activity than the wild type and some other viable MLPs (data not shown). Because of the low basal activity of the MLP in these experiments and the requirement for DNA replication for full activation of the MLP in the virus, we chose to insert these promoters into a nonessential region of adenovirus (the E1 region when using the complementing 293 cell line) and to monitor their transcription rates during the early and late phases of infection.

Figure 6 shows the structures of the plasmids used to construct the recombinant viruses for these experiments. β-Globin was chosen as a convenient assayable sequence since it lacks any homology to adenovirus sequences and the endogenous globin genes are not transcribed in 293 cells. After infection with either MLPG-wt, MLPG 13, or MLPG 14, nuclei were isolated at either 6 or 24 h postinfection. Run-on transcription in isolated nuclei was quantitated by dot blot hybridization to excess unlabeled DNA probes (Table 4). The values for transcription from the three sequences that were analyzed were compared by setting the values for each transcription unit in the MLPG-wt infection at unity. This allowed two comparisons to be drawn: the relative ability of the mutant MLP to be transcribed in

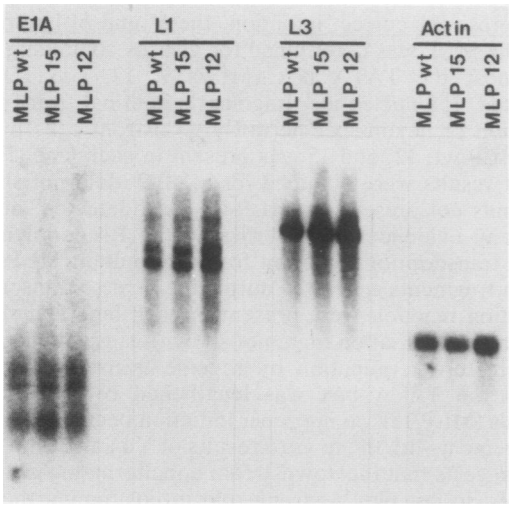

FIG. 4. Analysis of viral mRNA species after infection of HeLa cells with MLP-wt, MLP 12, and MLP 15. Poly(A)$^+$ RNA was isolated from infected cells 18 h after infection with 20 PFU of MLP-wt, MLP 12, or MLP 15 per cell, and 0.2 μg of denatured RNA was fractioned on 1% agarose gels containing 2.2 M formaldehyde as described in Materials and Methods. Following transfer to nitrocellulose, RNAs were hybridized to nick-translated E1A (2.55 to 3.72 m.u.), L1 (32.15 to 37.94 m.u.), L3 (50.97 to 60.12 m.u.), or actin DNA.

TABLE 3. Transcription of altered MLPs in isolated nuclei from infected cells[a]

MLP	Time postinfection (h)	Expt	% of wild-type transcription
4	15	A	150
	24	A	78
9	15	B	129
	24	B	82
12	19	C	128
		D	127
15	15	A	85
		B	63
	19	C	119
		D	136
	24	A	131
		B	36

[a] Experiments A and B were performed in HeLa cells and C and D in KB cells, all in spinner culture. The percent transcription compared with the wild type was calculated as follows: counts hybridizing to L1 and L3 sequences were added and normalized to those hybridizing to E1A sequences in both wild-type and mutant infections in the same experiment. Typical values are exemplified in experiment C: MLP-wt: L1, 18,000 cpm; L3, 71,000 cpm; E1A, 390 cpm. MLP 15: L1, 13,000 cpm; L3, 76,000 cpm; E1A, 331 cpm.

relation to its wild-type analog in the same genome and in relation to MLPG-wt in the parallel infection. Since conditions in each infection will not be identical, the latter comparisons should be corrected by using the E4 region transcription as a control for template copy number. When these comparisons were made, it was seen that MLP 14 had, on average, an approximately twofold reduction in transcription at the early time, rising to a sixfold reduction at the late time of infection. MLP 13, on the other hand, had only 1.4-fold and 1.9-fold reductions at early and late times, respectively. It is worth noting that the MLP-wt present in the E1 region had an activity some threefold lower than that of the normally positioned MLP in the same genome (data not shown).

DISCUSSION

We have developed a plasmid and virus system by which the adenovirus MLP can be replaced easily with mutant MLPs in order to examine the ability of these promoters to support a productive infection. The surprising result is that even though the MLP is highly regulated, this sequence was able to accept many different alterations without any phenotypic effect (Table 1). This is more surprising because the gene encoding the DNA polymerase is located on the other strand, opposite the MLP (2, 15). These studies also showed

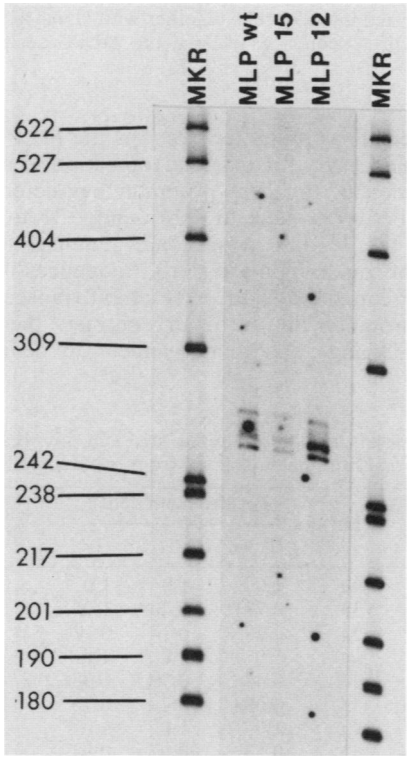

FIG. 5. Primer extension analysis of the 5′ ends of hexon-specific mRNAs produced after infection with MLP-wt, MLP 12, and MLP 15. KB cells were infected at 20 PFU/cell. At 19 h postinfection, cytoplasmic RNA was isolated, and 2.5 μg of poly(A)$^+$ RNA was annealed with the hexon-specific primer isolated from pHexP and then extended with reverse transcriptase. The reaction products were denatured and electrophoresed on a 6% acrylamide gel. Markers (MKR) were pBR322 plasmid DNA cleaved with *Hpa*II and end-labeled with [γ-^{32}P]ATP. The 257-nucleotide fragment is the slowest-migrating species. Sizes (in nucleotides) are indicated.

that the G sequences immediately downstream of the TATA box are amenable to extensive changes without phenotypic consequences, suggesting that the MLP and DNA polymerase sequence are very malleable in this particular region. Five of the mutant sequences could not function in place of the wild-type MLP, and two of them, MLP 13 and 14, were shown to have a transcriptional defect when assayed in the E1 region of the viral genome.

Structure of the wild-type and mutant MLPs. One of the aims of this study was to examine the importance of the nonalternating G residues bracketing the TATA box. The sequences are highly conserved in the MLPs of the adenovirus types 2, 5, 7, and 12 (43a; reviewed in reference 44), and they are not found in other adenovirus promoters. Ziff and Evans (55) proposed that the G-rich sequences downstream of the TATA box participate in the formation of a hairpin and loop structure necessary for the efficient initiation of transcription. Such a structure would expose S1-sensitive sites, as are found in plasmid constructs (16), in the MLP integrated in cellular chromatin (25), and in the viral chromosome late in infection (50). However, extensive point mutagenesis of this region did not affect the activity of the MLP in vitro (53), and such mutations would be expected to prevent the formation of this structure and the S1-sensitive site. From the results of subsequent transfection experiments, Yu and Manley (54) proposed a role for the S1-sensitive site during infection. In this model, the presence of the secondary structure and S1-sensitive site during the early phase would hinder transcription from the MLP. Activation of transcription of the MLP would occur after DNA replication and removal of this structure. However, our results suggest that this is not the case. Thus, MLP 15 and MLP 16 contain extensive mutations that would decrease the energy of formation of this hairpin, and they were normal (to the limits of our assays) at both early and late times, implying that the proposed structure and S1-sensitive site are not involved in regulation of the MLP in the virus.

The nonalternating G sequences have some similarity to sequences surrounding the TATA boxes of other eucaryotic promoters, such as chicken adult β-globin. It has been proposed that such flanking GC-rich sequences may act as a trap for the RNA polymerase (6) by increasing the melting temperature of the DNA in this area, but again the series of viable mutant MLP viruses (e.g., MLP 15) makes this model unlikely to hold true, at least for the MLP.

Lethality of MLP 13 and MLP 14 in the correct genomic location. Of the five lethal mutant sequences, MLP 13 and MLP 14 were chosen for further study because they contained the fewest numbers of transitions and useful sequence comparisons could be drawn with MLPs 12, 15, and 16, which contained as many or more transitions and yet could be incorporated into viable virus. The lethality exhibited by MLP 13 or 14 could arise from one or a combination of three sources: the mutations could interfere with transcription from the MLP, create lethal amino acid changes in the DNA polymerase, or disrupt the promoter for the essential IVa_2 gene. To begin to separate these three causes, viruses containing globin sequences driven by the respective MLPs and located in the nonessential E1 region were constructed (Fig. 6). Globin transcription was lowered by some sixfold for the MLP 14 construct at a late time in infection (Table 4). Thus, it is clear that MLP 14 does indeed have a transcriptional defect. Two of the five G-to-A transitions in MLP 14 occurred in one or more of the viable mutant sequences, leaving the adjacent G-to-A transitions at positions −35 and −36 and a single transition at −12 as candidates for the

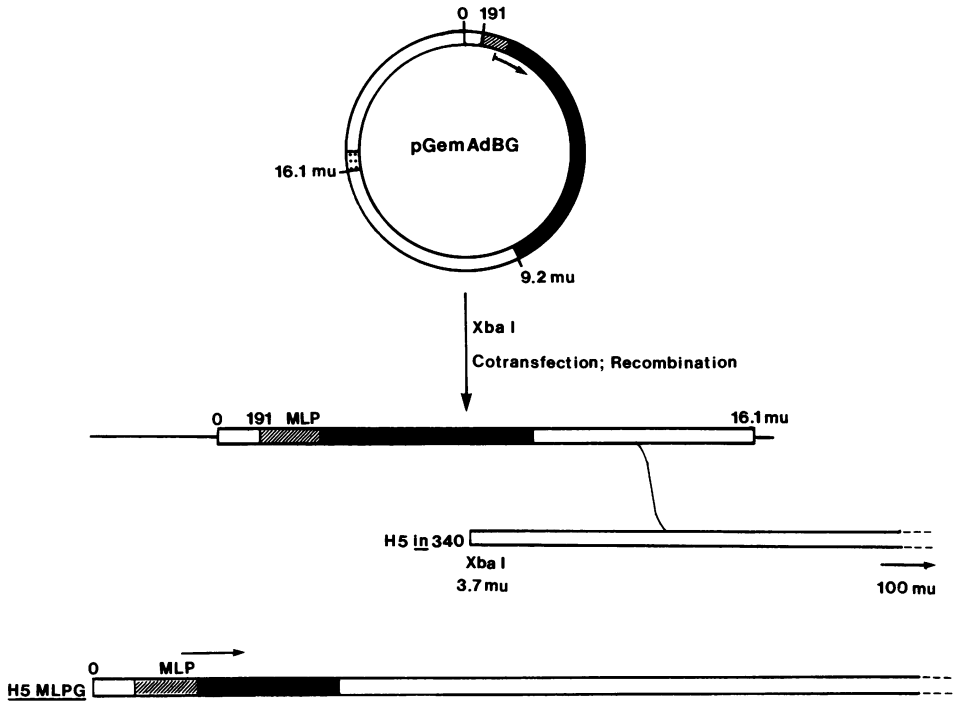

FIG. 6. Construction of viruses containing a second MLP in the E1 region of adenovirus. The promoter fusion plasmid pGemAdβG (Fig. 2) was digested with *Xba*I, which cuts in the polylinker, and this material was transfected into 293 cells together with H5*in*340 DNA, which was digested with *Xba*I and *Cla*I. Recombination between the homologous adenovirus sequences yielded the MLPG series of viruses containing an extra copy of the MLP in region E1.

transcriptional deficiency. MLP 13 had a reproducible twofold reduction in transcription at late times, and possible candidates for the responsible mutation are the adjacent G-to-A transitions at −37 and −36 and the single transitions at −14 and −18. None of these changes lies within known transcription-factor-binding sites.

Possible effects on the DNA polymerase. MLP 13 had only a twofold reduction in transcription when positioned in the E1 region. It seems unlikely that such a small change would be lethal in the normal genomic context, in view of the large amounts of mRNAs and polypeptides present late in infection. It is possible that the lethality of MLP 13 could be attributed, in part, to the specific changes occurring in the coding region assigned to the DNA polymerase (2, 15). However, all three codons which were altered in MLP 13 were altered in other viable viruses, although to different amino acids at two of the three sites and in different combinations in the various mutants.

While we cannot argue unambiguously for or against a role in lethality for the amino acid changes in the nonviable mutants, the most striking observation was the marked tolerance for amino acid change exhibited by the viable mutants. Each of the 12 amino acids which could be changed by bisulfite mutagenesis had been altered in one or other viable mutant, frequently to more than one possible alternative. This region of the adenovirus genome may be analogous to the overlapping p_{RE} promoter and *c*II gene of bacteriophage λ (52). In this region, critical areas of the p_{RE} promoter do not coincide with critical areas of the *c*II protein. Adenovirus may have evolved similarly, so that the regulatory region of the MLP does not overlap a part of the DNA polymerase in which primary sequence conservation is mandatory for function.

Possible effects on transcription from the IVa_2 promoter. The divergent IVa_2 promoter overlaps the MLP and shares sequences necessary for efficient transcription in vitro (35, 36). Dissection of the IVa_2 promoter has determined that sequences between −162 to −171 and −31 to −58, with respect to the MLP cap site, are required for positive regulation of transcription in vitro. Sequences downstream of the MLP cap site (+1 to +33) inhibit transcription from the IVa_2 promoter in vitro. In contrast, the sequences between −58 and +32 do not appear to be involved in

TABLE 4. Transcription of altered MLPs in the MLPG series of viruses in nuclei isolated from infected cells[a]

Time postinfection (h)	MLPG	Relative transcription			Ratio, globin/ [(L1 + E4)/2]
		Globin	L1	E4	
6	wt	1.0	1.0	1.0	
	13	0.79	0.40	1.05	1.09
	14	0.29	0.14	0.78	0.63
24	wt	1.0	1.0	1.0	
	13	0.44	0.79	0.98	0.50
	14	0.098	0.79	0.76	0.13
6	wt	1.0	1.0	1.0	
	13	0.53	0.97	0.94	0.55
	14	0.33	0.76	0.75	0.44
24	wt	1.0	1.0	1.0	
	13	0.42	0.70	0.79	0.56
	14	0.12	0.45	0.60	0.23

[a] Nuclei were isolated at the times indicated and treated as described in Materials and Methods. Autoradiographic exposures of labeled RNA hybridized to the various filters were scanned densitometrically, and the values for MLPG-wt were set at 1.0. Comparisons can be drawn between the values for MLPG transcription of globin compared with the average number of L1 counts (derived from transcription from the MLP) and E4 counts (driven by the E4 promoter at early times and from the MLP at late times).

control of the IVa_2 promoter, as determined by plasmid-mediated transfection (37). However, these experiments defined IVa_2 promoter elements when the simian virus 40 enhancer was present in *cis* and thus may be measuring promoter sequences necessary for enhancer-mediated transcription. Because the region between −58 to −31 does exert a positive effect on IVa_2 transcription in vitro, and the adjacent G-to-A transitions of MLP 13 and 14 lie in this region, we cannot rule out the possibility that these mutations may alter the balance of transcription from the MLP and the IVa_2 promoter and contribute to the lethal phenotype. On the other hand, mutant 14 was shown to have a reduced transcription rate from its MLP, and therefore it is unlikely that alterations in the IVa_2 promoter are solely responsible for its lethality.

Could the upstream G sequence be important for transcription factor binding? Footprinting of partially purified transcription factor IID (TFIID) to the MLP showed that these sequences are protected by this transcription factor from DNase I cleavage (41). On the other hand, methidium propyl-EDTA-Fe(II) footprinting showed that only the TATA box sequence itself was protected by TFIID. Similarly, the MPE Fe(II) footprinting of transcription factor USF showed binding to a 12-bp sequence centered at −58 (41). Thus, neither of the partially characterized transcription factors bind directly to the upstream G-rich sequence. However, binding of TFIID stabilizes the association between USF and its binding sequence (41), suggesting that the two transcription factors interact either by direct protein-protein interaction or by inducing changes in the DNA structure. If the latter is the case, alterations to the intervening DNA sequence in MLP 13 and MLP 14 may disrupt the normal propagation of this structural signal.

Use of virus reconstruction to study functions in the MLP region. The results presented in this paper demonstrate that a mutational analysis of the region surrounding the MLP can be conducted by attempting to reconstruct virus. Contrary to earlier expectations, the potential amino acid sequence of the DNA polymerase can be mutated variably and extensively without deleterious effects. It may prove to be similarly malleable in other areas of great current interest, such as the upstream factor-binding site and the various IVa_2 transcription signals. If this is so, mutagenesis and attempted replacement of mutations in virus may reveal details of transcriptional control not readily detected in vitro or in transient expression assays.

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