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The Initiator Element of the Adenovirus Major Late Promoter Has an Important Role in Transcription Initiation In Vivo

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Previous results showed that the structure and function of the adenovirus major late promoter (MLP) can be analyzed genetically in its correct location, despite its essential role in the viral life cycle. This genetic approach was extended to investigate the in vivo role of the initiator (INR), a transcriptional element that surrounds the start site of transcription. The analysis was designed to investigate if the INR is an alternative basal element to the canonical TATA box of the MLP, its relative importance in the functioning of the promoter, and if its function was affected by upstream activating elements. Accordingly, two different mutations in the INR were created and tested in the genome, either by themselves or together with mutations in the TATA box or one of the two upstream activating elements, the upstream promoter element (UPE) and the inverted CAAT box. The mutant viruses were examined first in one-step growth experiments, and then levels of late mRNA accumulation were measured by primer extension, transcription initiation was assayed in isolated nuclei, and viral DNA accumulation was determined by Southern hybridization. Neither mutation in the INR alone had any discernible phenotypic effects but when coupled to a phenotypically silent mutation in the TATA box gave rise to viruses with growth defects that were attributable to a significantly lowered rate of transcription initiation from the MLP. These results suggest that the INR plays a role in vivo and can act as an alternative basal element in the absence of a functioning TATA box. A virus with mutations in both the INR and the UPE, although viable, likewise had a severe deficiency in transcription, suggesting that the function of the INR is affected by that of the UPE. This contrasts with the previous report that a TATA box-UPE double mutation is not recoverable in virus. In addition, the virus with mutations in both the INR and the inverted CAAT box was phenotypically wild type, unlike the previously described TATA box-CAAT box double mutant, which had a severe transcription deficiency. Taken together, the present and previous genetic results can be interpreted as evidence that in the MLP, the TATA box and the UPE are the more important of the two basal and activating elements, respectively, but that the INR and CAAT can function in transcription initiation. We consider the role of the INR in the formation of the preinitiation complex and speculate on possible protein-protein interactions.

RNA polymerase II (pol II)-mediated transcription from eukaryotic promoters involves the assembly of a large number of general or basal transcription factors into a preinitiation complex (reviewed in references 13 and 54). Within this preinitiation complex, the only polypeptide that displays sequence-specific DNA binding is the TATA box-binding protein (TBP), which binds to the minor groove of the DNA helix (25, 32, 45). Most other general transcription factors associate with the promoter region of the DNA via nonspecific contacts with the phosphodiester backbone and/or by protein-protein interactions with TBP. Recent solution of the crystal structures of ternary complexes between TBP and the TATA box with either TFIIB (33) or TFIIA (19, 47) has given a clear picture of the multiple contacts formed between these proteins and with the TATA box. However, it has been known for several years that the DNA sequence surrounding the start site of transcription also plays an important role in the accurate and quantitative initiation of transcription from many cellular and viral genes (reviewed in reference 42). Indeed, for most of those pol II promoters that lack a TATA box, it is likely that the DNA sequence at the site of initiation constitutes the primary basal element in establishing the preinitiation complex (36, 43, 44, 55). Unlike the TATA box, which has a strict consensus sequence, the initiator element shows no single consensus, although certain motifs have been established (22, 40), and recent evidence demonstrates that there are sequence-specific polypeptides capable of binding to particular initiation regions (15, 37, 40, 50).

The adenovirus major late promoter (MLP) is one of those promoters for which the evidence is strongest that the initiation region contains a specific promoter element important for accurate and quantitative transcription. The MLP, which is responsible for the expression of mRNAs for most of the late structural proteins, was originally thought to be a relatively simple promoter with a canonical TATA box sequence and an upstream activating element, the upstream promoter element (UPE) (39). Two lines of evidence led to the idea that the MLP also contains an initiator element. First, Concino et al. showed that a single point mutation at the start site (+1) reduced the level of transcription in vitro (14). This observation was subsequently supported by transient transfection experiments using plasmids containing the MLP and a chloramphenicol acetyltransferase gene reporter (29). The early studies in vitro also showed that there was some functional redundancy, or interaction, between the factors that bound to the TATA box and the initiation site, because double mutations displayed a greater reduction in transcription than did single mutations in either element alone (14). The second line of evidence came from analysis of the promoter region of the gene encoding terminal deoxynucleotidyltransferase. This gene lacks a TATA box, but functional analysis showed that the region surround-

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ing the start site was important for quantitative transcription (43). Upon inspection, it was apparent that the sequence bore a striking similarity to the start site region of the MLP, and the two sequences were functionally interchangeable (43). Subsequently, several different polypeptides that bound to the initiation region of the MLP were identified (15, 18, 37, 38) and were shown to play a role in transcription either in vitro or in transient transfection (15).

From these lines of evidence, it is clear that the MLP does indeed contain an initiator element, now referred to as the INR (43), but its role in the complete promoter in the correct genomic context has not been established. It is important to compare and contrast results gained from transient transfections and in vitro reactions using mutant forms of a particular promoter to those obtained from studies in the viral genome, because they are not always congruent. This has been demonstrated clearly with the MLP (34, 35) and recently with the E2 early promoter (46). To address the biological role of the INR, we used the mutagenesis and viral reconstruction strategy developed previously to study the MLP in its native context (34). In this experimental system, substitution mutations are created in the desired element of the MLP, and they are then placed by overlap recombination into the correct position in the viral genome. The phenotype of the mutations can be assessed in the course of a normal viral replicative cycle. Using this genetic system, we showed (34, 35) that the MLP is considerably more complex than previously suspected. Specifically, the UPE, shown to be of prime importance in transient assays (29, 31), is not essential for high-level transcription from the MLP in vivo (34), the inverted CAAT box can substitute for the absence of a functional UPE (34), and the TATA box is important but not essential for promoter function (35). Preliminary evidence (35) also suggested that the INR was likewise not essential, but this may be caused by functional redundancy with the TATA box. In this study, we confirmed these initial findings by using combinations of mutants in the INR, TATA box, and upstream activating elements. We show that single and multiple mutations in the INR have no effect on transcription from the MLP but, when coupled with a single point mutation in the TATA box, have a profound effect, as measured both in the viral replication cycle and more directly at the level of transcription. A further interesting interaction is suggested by the observation that a double-mutant genome with mutations in the UPE and in the INR is severely compromised in its growth and transcription abilities. This finding suggests that there is some interaction between USF, which binds to the UPE (10, 31, 39), and the protein(s) that binds to the INR, and we discuss the possible role of the TBF-associated factor $TAF_{II}150$ (49, 50) in this interaction.

MATERIALS AND METHODS

Mutagenesis and mutant virus construction. All virus mutants were created using the M13 *dut ung* mutagenesis protocol of Kunkel (27), followed by a two-step reconstruction of the adenovirus genome, as described in detail previously (34). The mutational oligonucleotides were designed so that there was no change to the amino acid sequence of the DNA polymerase, encoded on the strand complementary to the MLP transcribed strand. Oligonucleotides used in the mutagenesis were as follows:

TATA30, 5′ (−20)CC CCC TTT TAc(−30) AGC CCC CC(−38) 3′
Start-1, 5′ (+10)G GAA GAG AGc(+1) GAG GAC GAA CGC C(−13) 3′
INR-5, 5′ (+16)GC GAT GCt GAg GAG tcg(+1) GAG GAC GAA CGC(−12) 3′

The oligonucleotides are presented so that the reading frame of the DNA polymerase can be deduced, and the mutations are shown in lowercase. The triple mutation in INR-5 uses one of the alternative codons for serine. The mutagenesis of the UPE and the inverted CAAT box to yield the USF0 and CCCAT mutants has been described previously (34). Double mutations in transcription elements were obtained by sequential use of individual oligonucleotides. After identification of the M13 clone with the desired mutation(s), the MLP region was exchanged with that of plasmid pMR2, which contains adenovirus type 5 (Ad5) DNA sequences extending from the left-hand end to bp9523. Human A549 cells were cotransfected with the mutated plasmid and *Pae*R7I-digested DNA-protein complex (DNA-PC), derived from purified virus LLX1 by standard procedures (52). LLX1 is a phenotypically wild-type chimera of Ad5 and $Ad2^{+}ND1$ (2). Overlap recombination between the plasmid sequences and the cut DNA-PC yielded virus in all cases. After plaque purification of the individual mutant viruses, the region immediately surrounding the MLP was recloned and sequenced. All sequences were as expected from the mutagenesis.

Measurement of viral replication cycles. Human A549 cells in 35-mm-diameter dishes were grown to confluency in Dulbecco modified Eagle medium with 10% supplemented calf serum (HyClone, Logan, Utah) and infected at a multiplicity of infection (MOI) of 10 fluorescent focus units (FFU) per cell. After adsorption, the cells were incubated at 37°C, and at intervals, dishes were removed from the incubator and the entire contents were frozen. Following two more cycles of freezing and thawing, the samples were titrated by fluorescent focus assay (28), and the total numbers of viruses per cell was calculated. Viral DNA accumulation during the replicative cycle was measured by Southern hybridization on a slot blot apparatus as described previously (34).

Reversion analysis and marker rescue. An early passage stock of mutant virus TATA30::start-1 was plaque purified twice, and the viruses in five individual plaques were isolated. These five isolates were then passaged twice on A549 cells, and the five stocks were retitrated. Plaques arising early and displaying a large wild-type morphology were identified, and the virus in them was isolated. One such clone, designated rev 2, was further plaque purified and amplified. Intracellular adenovirus DNA was isolated by using a modification of the Hirt technique, and the MLP region was cloned and sequenced. Marker rescue of the growth deficiency of USF0::start-1 by plasmid DNAs was performed by using techniques described earlier (51). Briefly, DNA-PC was isolated from purified virions of USF0::start-1. Because USF0::start-1 replicates poorly, the virions were prepared from five 175-cm^2 flasks of infected A549 cells. A549 cells in 35-mm-diameter dishes were cotransfected with 100 ng of this DNA-PC and 300 ng either of plasmid pMR2 or of a plasmid pDQ3, containing only the region immediately surrounding the MLP from the *Xho*I site at bp 5788 to the *Hin*dIII site extending through bp 6246 (Ad5 numbering system). The plasmids were either uncut or cut with various restriction enzymes prior to the transfection. After DNA precipitation and adsorption, cells were washed and overlaid with a liquid medium as described earlier (51). After incubation at 37°C for 7 days, the yields were harvested and titrated by plaque assay. Individual large plaques were identified, and the virus DNA was characterized for the presence of restriction sites diagnostic for the MLP region of the rescuing plasmid DNA. These diagnostic sites are a 6-bp *Eco*RI site, derived from pMR2 (34) and inserted in the first late leader, and a 6-bp *Sna*BI site derived from pDQ3 and inserted in the UPE (33a). Neither insertion, by itself, confers any recognizable growth phenotype.

Primer extension assays. A549 cells were grown to confluency in 6-cm-diameter dishes and infected with the various viruses at an MOI of 10 FFU per cell. The infected cells were harvested in the late phase of the replicative cycle, and total intracellular RNA was isolated by using either of two commercial extraction methods (Tel-Test Inc., Friendswood, Tex.) based on the one-step procedure of Chomczynski and Sacchi (11). If RNAzol B was used, infected cells were washed with phosphate-buffered saline (PBS) before suspension in the guanidinium-containing reagent, but if RNA STAT-60 was used, the PBS washing step was omitted. RNA samples were treated with RNase-free DNase (Promega, Madison, Wis.), and the amounts of RNA recovered were measured by A_{260}. Equivalent amounts of RNA were examined by hybridizing a 5′-end-labeled primer complementary to the first leader of the major late transcription unit (MLTU). The duplex was extended by using avian myeloblastosis virus reverse transcriptase (Promega) as described in detail previously (34). Extended DNA products were separated on a 6% polyacrylamide sequencing gel. The amount of labeled product was quantitated by densitometry of autoradiographs exposed within the linear range of intensity. At the relative levels of RNA and primer used in the primer extension assays, in which the primer is in excess, the quantity of extended product is directly proportional to the level of steady-state mRNA. Although no internal control for variability in RNA extraction from different batches of infected cells was attempted, the experiments were repeated at least three times with the various mutants, and the results were quantitatively similar.

Nuclear run-on assays. The techniques for preparation of nuclei, nuclear transcription, and analysis of the labeled nascent RNA by hybridization to M13 single-stranded probes on nylon membranes have been described in detail previously (34) and were used with some modifications based on a published protocol (20). Briefly, A549 cells, grown in 175-cm^2 flasks, were prepared 1 day before infection. The confluent monolayers were infected at an MOI of 10 FFU per cell with the various mutants and incubated for 20 h at 37°C. The infected monolayers (approximately 5×10^7 cells) were washed three times with ice-cold PBS and were resuspended in 4 ml of Nonidet P-40 (NP-40) lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40). The cells were kept on ice for 5 min and centrifuged for 5 min at 500 rpm in a model CR6000 IEC refrigerated centrifuge, and the nuclear pellet was resuspended in 4 ml of NP-40 buffer. They were centrifuged again, and the pellet was resuspended in 100 μl of glycerol storage buffer (50 mM Tris-HCl [pH 8.3], 5 mM $MgCl_2$, 0.1 mM EDTA, 50% glycerol). Approximately half of each of the nuclear samples was used immediately for the transcription reaction, which was essentially as described

TABLE 1. Mutations in the elements of the MLP

Virus	Sequence of MLP element[a]		
	UPE	TATA box	INR
Wild type	GGCCACGTGACC	TATAAAA	TCCTCACTCTCTTCC
Start-1			TCCTCgCTCTCTTCC
INR-5			TCCTCcgaCTCcTCa
TATA30		TgTAAAA	
USF0	aGCtACaTGgCC		
TATA30::start-1		TgTAAAA	TCCTCgCTCTCTTCC
TATA30::INR-5		TgTAAAA	TCCTCcgaCTCcTCa
USF0::start-1	aGCtACaTGgCC		TCCTCgCTCTCTTCC

[a] The UPE is a typical E box and contains the site of highest affinity, RYCACGTGRY (1), the TATA box is a canonical sequence, TATA(T/A)AA (3), and the region of the INR is shown as a predominantly pyrimidine stretch, with a single purine residue at +1. It conforms to the consensus PyPyAN(A/T)PyPy (22). Mutations are indicated in lowercase.

before except that incubation was at 25°C for 25 min. Following incorporation of [^{32}P]UTP (100 μCi, 800 Ci/mmol; NEN), RNA was isolated and samples containing 6×10^6 cpm were hybridized by standard procedures to nylon membranes (Protran; Schleicher & Schuell). The membrane was prepared on a slot blot apparatus to which had been added individual samples of 10 μg of a set of single-stranded DNAs from M13 clones. Hybridization was at 65°C for 48 h, followed by extensive washing of the membrane and exposure in a PhosphorImager screen (Molecular Dynamics). Quantitative analysis was performed on the images as described in the legend to Fig. 5.

RESULTS

Creating mutations in the INR element of the MLP. Previous evidence showed that the A residue at +1 plays an important role in accurate and quantitative transcription from the adenovirus MLP in vitro (14). The first mutation to be made therefore was a transition from A to G (start-1 in Table 1). This is the only single-point mutation possible at the start site which conserves the amino acid sequence of the DNA polymerase encoded on the strand complementary to the MLP. However, because the codon encodes a serine, it is possible to replace the triplet with a completely different triplet. This more radical change might completely disrupt the binding of an INR-specific protein, whereas a single base pair alteration might not. Accordingly, a mutation was made in which these residues and two others further downstream were changed while still maintaining the primary amino acid sequence of the DNA polymerase (INR-5 in Table 1). As well as creating these two mutations in an otherwise wild-type background, they were also combined with previously described mutations in the TATA box or in one or the other of the two functionally redundant upstream elements, the UPE and the inverted CAAT box. The M13 mutant MLP sequences were transferred to pMR2 and used in overlap recombination with *Pae*R7I-digested genomic DNA from virus LLX1. All cotransfections yielded virus, but the plaque sizes of all doubly mutant viruses, except CCCAT::INR-5, were much smaller than those with the single-mutant sequences, and the individual infected cells displayed a distinctive cytopathic effect typical of MLP mutants with deficient replicative cycles (data not shown). Following plaque purification and confirmation that the isolates contained the expected mutations in the MLP, high-titer stocks were prepared and analyzed.

Replicative cycles of viruses with mutations in the INR and TATA box. The preliminary evidence of the transfection plaque assay suggested that the replicative abilities of viruses with single mutations in the INR were similar to those of the wild type, while those with mutations in both the INR and the TATA box were deficient. Previous work (34, 35) has shown that a deficiency in viral replication in MLP mutant viruses is quantitatively related to the reduction in MLP transcription. To examine this more closely with the new mutants, human A549 cells were infected with the individual viruses, and samples were taken at intervals to measure viral replication in one-step growth curves (Fig. 1). Viruses TATA30, INR-5, and start-1 all replicated with efficiency equal to that of the wild type, with very similar eclipse periods and final viral yields. The slightly higher yield of the wild-type sample at 26 h postinfection was not repeated in other experiments and is probably attributable to experimental error. These results suggest that the mutations in the INR element do not affect the transcriptional abilities of the promoter, either because the binding of transcription factors to the INR is not altered by the mutations or because the promoter has redundant compensating mechanisms for the establishment of the preinitiation complex. In contrast, the replication of mutant viruses TATA30::start-1 and TATA30::INR-5 was considerably reduced compared to that of the single-mutant viruses, the final yield being approximately 10-fold lower at the final time point. This replicative deficiency correlates with the observed smaller plaque size of the double-mutant viruses. Taken together, these results strongly suggest that the two elements, the TATA box and the INR, can compensate functionally for each other. Note also that the deficiency in replication of the double mutants is genetic evidence that the mutations in the DNA sequences do indeed affect the binding of the respective factors in vivo.

Genetic evidence for an interaction between the INR and the UPE. Early biochemical evidence had suggested that there is an interaction between the factors that bind to the UPE and the TATA box in the adenovirus MLP (39). Previous genetic evidence corroborated this suggestion, because it was impossible to create viruses with combined mutations in the UPE and the TATA box (35). The question then arose as to whether the same held true for the alternative basal element, the INR. To test this, a double mutant virus, USF0::start-1, was created. This mutant contains a four-point mutation in the UPE, which renders it unable to bind the activating factor USF (34), and the single-point transition at the start site. Although it is viable, the mutant virus displayed a much smaller plaque size than the wild type, and in one-step growth experiments it showed an approximately 15-fold reduction in final titer compared with either of the single mutants (Fig. 2). The single mutants USF0 and start-1 replicated as efficiently as the wild type (34) (Fig. 1). The genetic analysis thus suggests that there is some form of interaction, either direct or indirect, between the factors

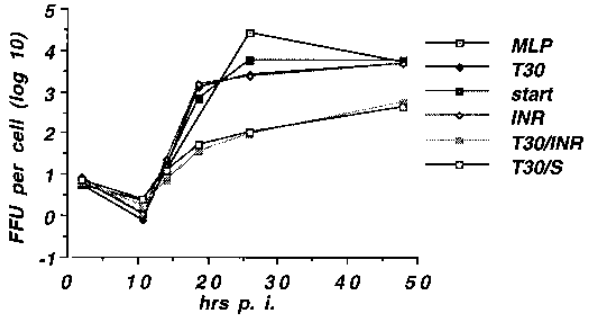

FIG. 1. Replication of viruses with mutations in the MLP TATA box, the INR, or both. A549 cells were infected with the various viruses, harvested at intervals, and titrated by fluorescent focus assay as described in Materials and Methods. The number of FFU per cell was calculated and plotted on a logarithmic scale as a function of time postinfection (p.i.) in hours. Abbreviations for mutant names: T30, for TATA30; Start, Start-1; INR, INR-5; T30/INR, TATA30::INR-5; T30/S, TATA30::start-1. MLP is a recombinant between LLX1 and a plasmid containing the wild-type MLP.

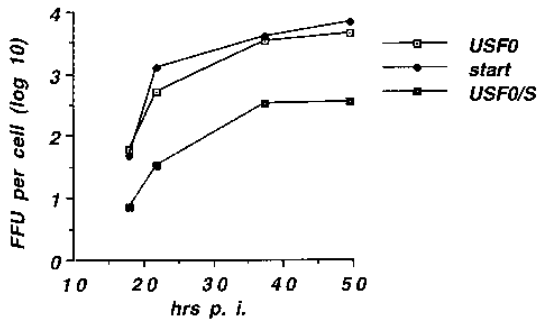

FIG. 2. Replication of viruses with mutations in the INR, the UPE, or both. Methods were as described in the legend to Fig. 1. USF0/S USF0::start-1; p.i., postinfection.

that bind to the UPE and to the INR. In contrast to the phenotype of the USF0::start-1 double mutant, the virus containing mutations in both the inverted CAAT box (CCCAT) and the INR had no detectable replication deficiency (data not shown and Fig. 4). This is in contrast to the phenotype of the CCCAT::TATA27 double mutant, which has the most deficient phenotype of any of the viable viruses created so far (35). These results suggest that the interactions between the INR and the upstream elements are distinct and may be restricted to the factor that binds to the UPE. The implications of these observations for the interactions between the factors that bind to basal and activating elements will be considered further in Discussion.

The deficient phenotypes of mutant viruses TATA30::start-1 and USF0::start-1 are caused by the known mutations in the MLP. It is always a concern in the construction of new viruses, involving several DNA manipulations and several transformations into both prokaryotic and eukaryotic cells, that the genomes of the new viral isolates might contain some unsuspected mutations in regions other than those being investigated. Although the sequences of the region around the MLP region were always as expected in all of the new isolates (data not shown), these observations did not exclude the possibility that other hidden mutations actually conferred the observed phenotype. Two strategies were used to test this formal possibility. Both depended on the viral replication deficiencies of TATA30::start-1 and USF0::start-1. This deficiency allows the overgrowth of virus with wild-type replication abilities. The first strategy used reversion analysis of TATA30::start-1. Five freshly plaque-purified stocks of TATA30::start-1 virus were passaged twice in human A549 cells. After the second passage, the yields were titrated by plaque assay, and the plates were examined for plaques that arose early and in which the infected cells displayed a wild-type cytopathic effect. A single large plaque isolate was observed. The region surrounding the MLP was cloned and sequenced, and the virus was found to contain a change at the TATA box, restoring the wild-type sequence TATAAAA while retaining the start-1 mutation. This result proves beyond reasonable doubt that the phenotype of TATA30::start-1 is caused by the mutations knowingly incorporated into the MLP. A second strategy was used with USF0::start-1. If the replication deficiency is caused by the mutations in the MLP, it should be possible to select for recombinants with wild-type phenotypes following cotransfections of mutant viral DNA with plasmids containing wild-type MLP regions. Human A549 cells were cotransfected with full-length DNA-PC from USF0::start-1 and two different plasmids, pMR2 and pDQ3. The former contains wild-type adenovirus sequences extending from the very left-hand end of the genome to bp 9523 and a diagnostic 6-bp *Eco*RI site inserted into the sequence encoding the first leader of the MLTU (34). Titration of the viral yields from this cotransfection showed numerous large plaques, while a control transfection with the DNA-PC of USF0::start-1 did not. Furthermore, the genomes isolated from the large-plaque virus contained the extra *Eco*RI site. Thus, the mutant phenotype can be rescued by pMR2 DNA, and the causative mutations must lie within the first 9,523 bp of the virus. To narrow the location further, a cotransfection with plasmid pDQ3 was performed. This plasmid contains wild-type sequences from the *Xho*I site at bp 5788 to the *Hin*dIII site at bp 6241 and a diagnostic 6-bp *Sna*BI site inserted into the UPE at bp 5990. Most recombinants should contain this diagnostic site because it is tightly linked to the wild-type INR sequence (Fig. 3). Again large plaques were observed among the yield from the cotransfection, and individual cells in them had the characteristic cytopathic effect of wild-type-infected cells, while the small plaques had cells with mutant-infected morphology. Thus, a small 459-bp wild-type fragment in pDQ3 DNA can rescue the deficiency in USF0::start-1. A single isolate was examined and proved to have the extra *Sna*BI site. Taken together, the marker rescue results show that the phenotype of USF0::start-1 is conferred by the mutations intentionally placed in the MLP.

Primer extension analysis of viral RNA from mutant-infected cells. The data from the replication curves show that only the double mutants have a deficiency in virus accumulation. Because the reversion and marker rescue analyses demonstrate that this deficiency can be attributed unambiguously to the mutations present in the MLP, it is a reasonable presumption that the mutations have indeed affected the efficiency of transcription initiation. An alternative possibility is that the mutations at the start site have altered the precise sequence of the 5′ end of the primary RNA transcript in such a way as to affect the stability, processing, or transport of either the transcript or the mRNA product. A direct way to test this possibility is to perform primer extension analyses on the mRNAs elaborated at late times in infection with the various mutant viruses. This analysis will provide information about the retention of the extreme 5′ end of the primary transcript, and provided that the primer in the initial annealing step is in excess,

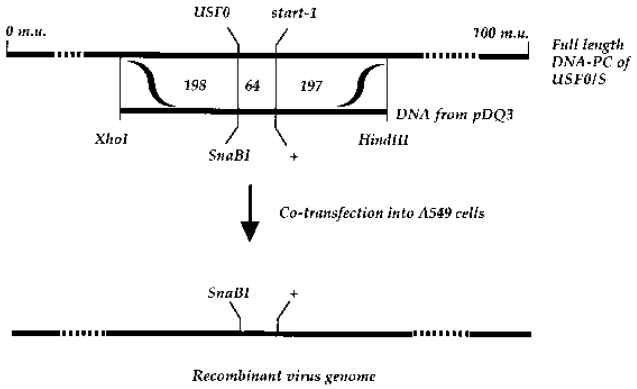

FIG. 3. Strategy for marker rescue of USF0::start-1 with DNA from pDQ3. The techniques for marker rescue and analysis of recombinant viral genomes are given in Materials and Methods. pDQ3 contains adenovirus sequence from the *Xho*I site at bp 5788 through the *Hin*dIII site at bp 6241. Recombination (curved lines) must occur within the 197 nucleotides to the right of the start-1 mutation located at bp 6049 and will occur more frequently (198/198 + 64) within the 198 nucleotides to the left of the first base pair change in the USF0 mutation at bp 5986. The *Sna*BI insert disrupts the UPE and thus is functionally equivalent to the USF0 mutation (33a). m.u., map units.

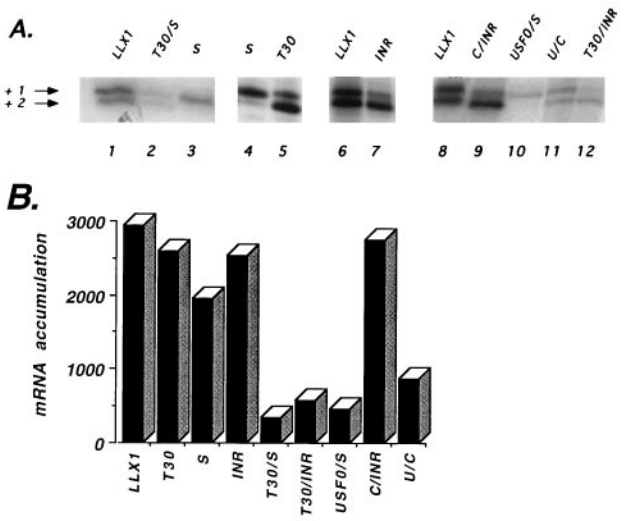

FIG. 4. Primer extension analysis of RNA isolated from A549 cells infected with various mutant viruses. (A) RNA was isolated from infected A549 cells during the mid-late phase of infection (18 to 24 h postinfection). Primer extensions were performed as described in Materials and Methods, using a primer whose 5′ end is at bp 6074, located in the first leader of the MLTU. The extension products are 36 and 35 nucleotides long (+1 and +2, respectively). Minor bands corresponding to RNA transcription initiations at −3 and −4 were visible on the original autoradiographs but have been excluded from this composite figure. Lanes 1 to 3, 4 and 5, 6 and 7, and 8 to 12 are taken from different samples of infected cell RNA and were processed on different occasions. Abbreviations for mutant names are as in Fig. 1, plus S for start-1, C/INR for CCCAT::INR, and U/C for USF0-CCCAT (34). (B) Various exposures of the autoradiographic images shown in panel A were scanned with a densitometer, and values in the linear range were tabulated. Values, in arbitrary units, were normalized as follows: values for lanes 1 to 3 and 6 to 12 were normalized using those for LLX1 (lanes 1, 6, and 8). Then the values for lanes 4 and 5 were normalized to the adjusted value of lane 3.

this assay also allows quantitation of the total amount of late viral mRNA present in the infected cell.

Human A549 cells were infected at an MOI of 10 FFU per cell, and the total intracellular RNA was isolated. Following hybridization to a DNA primer internal to the first leader sequence common to all late messages, the RNA-DNA hybrid was extended with reverse transcriptase and the products were displayed on a sequencing gel. The efficiency of mRNA synthesis in all of the single mutants was very similar to that of the wild type (Fig. 4A). In contrast, the efficiency of mRNA synthesis in the double mutants TATA30::start-1, TATA30::INR-5, and USF0::start-1 was considerably lower. Quantitative measurements (Fig. 4B) showed that the levels of primer-extended products in the single-mutant RNA samples were no more than a third lower than that of LLX1. Although the level of mRNA from start-1-infected cells was some 30% lower in the experiment shown, this reduction was not observed in samples obtained from three other experiments. On the other hand, extended products from TATA30::start-1 and TATA30::INR-5 were 6.7- and 4.5-fold lower than the averages from the respective single mutant-infected cells. These quantitative differences are similar to those observed in the replication curves shown in Fig. 1. The level of extension products in the double mutants USF0::start-1 and USF0-CCCAT also were much lower than those of LLX1 (6.3- and 3.4-fold, respectively), consistent with the results in Fig. 2 and published previously (34). The precise start sites of the mRNAs in the various mutant-infected cells showed some minor quantitative changes in the relative levels of transcripts started at +1 and +2, but there was no major change, for example, to an entirely new start site (data not shown). We and others (23) have shown that a minority of MLP primer extension products extend to the −3 and −4 positions. These minor species were observed in all mutant infections (data not shown). However the results in Fig. 4A also show that RNA samples from mutants containing the INR-5 mutation had a predominance of +2 products, while those from mutants containing the start-1 mutation had a predominance of +1 products. We do not know if this reflects the true 5′ structure of the respective mRNAs or a difference in the ability of the reverse transcriptase to extend to the end of mRNAs with the different sequences expected of INR-5 and start-1. Despite this uncertainty, it is clear that the steady-state level of viral mRNA is directly correlated with the different replicative abilities of the various mutants.

Nuclear run-on assays to measure RNA transcription initiation. The data from the primer extension assays are most easily interpreted to mean that mutants defective in viral replication have a corresponding deficiency in transcription initiation, but alterations to RNA processing, transport, and stability are not ruled out. A recent report has shown that transport of adenovirus mRNAs is correlated with rate of transcription (53), and so it is necessary to try to estimate transcription initiation directly, using a nuclear run-on assay. Nuclei were prepared from 5×10^7 A549 cells infected with the various mutants, and [^{32}P]UTP was incorporated in vitro. RNA was isolated and hybridized to a set of single-stranded DNA probes of adenovirus sequences cloned in M13 (Fig. 5A), and the counts bound to each probe were quantitated with a PhosphorImager (Fig. 5B). The levels of hybridization, and thus of incorporation in nuclei, of the RNA from cells infected with the double mutants TATA30::start-1 and TATA30::INR-5 were significantly lower than those in the wild-type- or single-mutant-infected cell nuclei. The reduction in transcription in the L1 region for TATA30::INR compared to the average of either single mutant was 5.4-fold, and for TATA30::start-1 it was 3.2-fold. For L4, the values were 30- and 9.1-fold, respectively. The apparent difference between the reductions in the two late regions was not examined further, although it would be interesting to see if this reflected a greater degree of attenuation in the mutant MLTU. Nevertheless, the results of the nuclear run-on assays, taken together with those from the quantitative primer extension assays, suggest that the replication deficiency of the double mutants can be attributed to a lowering in transcription initiation.

DNA accumulation in mutant-infected cells. Although the mutations in the MLP were designed so that there were no changes to the amino acid sequence of the DNA polymerase, it was important to demonstrate that the levels of viral DNA synthesis were unaffected by the alteration to MLP transcription in the double mutants. The dependence of MLP transcription on active DNA replication is well known (48), but it is conceivable that there are also feedback mechanisms to coordinate the two processes in the infected cell. Accordingly, viral DNA was extracted from infected cells by a modified Hirt procedure and the amounts of accumulated DNA were measured by Southern hybridization using a slot blot apparatus (Fig. 6A). Quantitation of the counts hybridized showed no substantial differences in timing or total accumulation of viral DNA (Fig. 6B). Thus there is no evidence of mechanisms to regulate the rate of DNA replication either upward or downward in those viruses with lowered MLP transcription. Thus measurements of transcription are not confounded by changes to DNA replication rate.

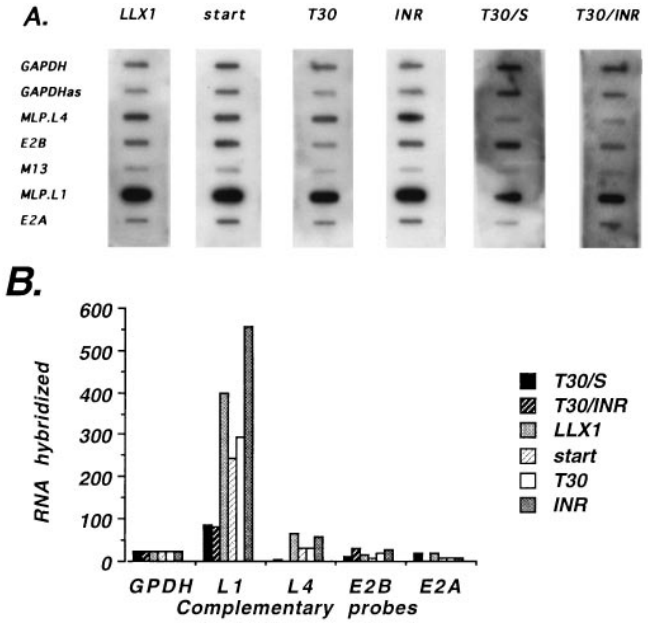

FIG. 5. Transcription in isolated nuclei from cells infected with various viruses. Experimental details are given in Materials and Methods. The data are from a single experiment in which six different viruses were used to infect A549 cells. (A) Individual filters with multiple slots containing single-stranded DNA from M13 clones were hybridized to RNA isolated from nuclei of cells infected with the various viruses. The M13 clones are as follows: GAPDH and GAPDHas are complementary to sense and antisense orientations, respectively, of the 1.3-kb *Pst*I fragment of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (17); MLP.L4 and E2A are complementary to the Ad5 late 100-kDa gene and early E2A gene, respectively, between the *Sma*I site at bp 23039 and the *Bgl*II site extending through bp 23917; MLP.L1 and E2B are complementary to the Ad5 L1 region and the E2B region, respectively, between the *Hin*dIII sites at bp 11565 and extending through bp 13651; M13 is M13mp18. Abbreviations for mutant names are as in Fig. 1. (B) Labeled RNA present on the filters was quantitated by PhosphorImager analysis, and the data (in arbitrary units) were normalized as follows. For each filter, the values for nonspecific hybridization to M13mp18 were subtracted from the values of the other slots. Then the values for hybridization to GAPDH were adjusted to those of the TATA30::start-1 filter, and the values for the other slots in each of the other filters were adjusted accordingly. The original values for GAPDH in the different filters varied less than 2-fold, and the ratios of GAPDH to GAPDHas varied between 1.4- and 2.5-fold. Values are plotted on an arithmetic scale.

DISCUSSION

Although the structures of many of the components of the preinitiation complex bound to eukaryotic pol II promoters are now known at atomic resolution (reviewed in references 4 and 21) and the centrality of TBP binding to the TATA box is established (reviewed in references 13 and 54), the role of the alternative basal element the INR is much less clearly defined. The importance of the INR in those promoters that lack a TATA box has been demonstrated (30, 43), and it seems reasonable to propose that its main role is to aid in recruiting TBP to the preinitiation complex (30, 44, 55). It is much less clear why some promoters have both a TATA box and an INR, although there are several different possibilities. These include functional redundancy, in which alternative basal elements behave in either different or similar manners to effect the same outcome; the integration of two different activating signals, mediated via the factors bound to the two elements, to achieve maximal expression; or a quantitative increase in the affinity (or stability) of the preinitiation complex for the promoter. In addition to uncertainty about its functional role, it is also not clear if the INR is a unique element, like the TATA box, or a collection of elements with different consensus sequences. Finally, the identification of the polypeptides that bind to the INR is not clearly established. Several different candidates have been described (15, 18, 24, 37, 38) as binding to the loose consensus PyPyAN(A/T)PyPy, defined by Javahery et al. for one class of initiator elements, the INR sensu stricto (22).

One of the prime examples of a promoter containing both basal elements is the adenovirus MLP (14, 29, 43). The purpose of the present investigation was (i) to determine if the INR played a role in transcription in the correct genomic context in vivo, (ii) to establish its importance compared with the TATA box, and (iii) to determine if it played a role in activation by the transcription factors that bind the two upstream activating elements, the UPE and the inverted CAAT box. Previous work had demonstrated the utility of this genetic approach (34, 35), and accordingly a set of viruses with mutations in the INR and in other elements was created (Table 1).

Both of the mutations in the INR result in a change from the consensus, but neither has a phenotype by itself. This could result if the mutations had no effect on the binding of the protein(s) that normally recognizes the INR or if the lowered binding is masked by strong binding of TBP to the TATA box. The results in Fig. 1 show that the latter possibility is the more likely, because viruses containing mutations in both the INR and in the TATA box have a severely deficient replication ability that can be traced to a lowered rate of transcription initiation in the double mutants (Fig. 5). These results demonstrate both that the mutations in the INR sequence had an effect on the binding affinity to the INR and that the INR can play a role in transcription in vivo. It should be noted that INR-5, which has a 5-bp change from the wild-type sequence, fortuitously creates a sequence centered at the A residue at +3 that conforms to five of the six nucleotides of the INR consensus. It was possible that this change could compensate for

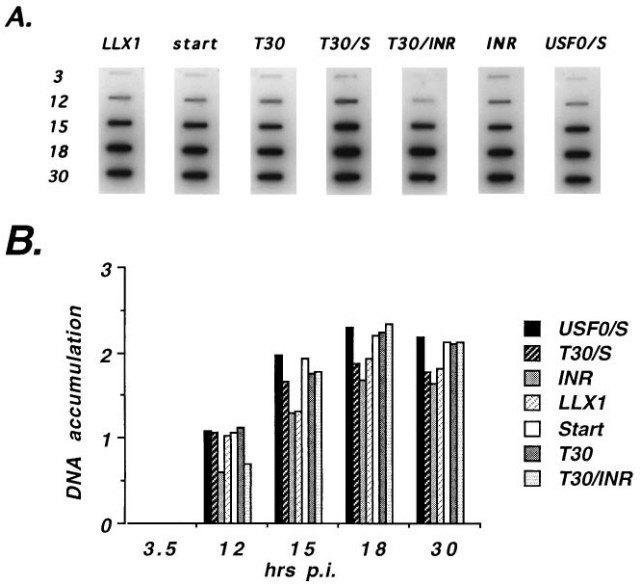

FIG. 6. Viral DNA accumulation in cells infected with various mutants. The data are from a single experiment in which seven different viruses were used to infect A549 cells. (A) Intracellular viral DNA was isolated at intervals from infected A549 cells by a modified Hirt technique. Samples were loaded onto a slot blot apparatus and probed with the right-hand-end *Bam*HI fragment of LLX1 DNA, labeled by random priming. The numbers on the left refer to hours postinfection. (B) The filter was analyzed with a PhosphorImager. Values (in arbitrary units) were normalized to those of the 3.5-h samples, presumed to be equivalent to values for the input viral genomes, which were set to 1 U. Values are plotted on a logarithmic scale. Abbreviations for mutant names are as in Fig. 1 and 4.

the intended loss of INR function at +1. However, the very similar results obtained with the double mutations TATA30::start-1 and TATA30::INR-5 in all replication and transcription assays strongly suggest that INR-5 is as defective as start-1.

The results are less clear-cut in determining which of the two basal elements is the more important, because the relative change in affinity caused by the different mutations is unknown. However, it should be noted that the mutant TATA0, with two point mutations in the TATA box, has a deficient phenotype (35), whereas the INR-5 mutation with five changes to the INR has none. In transient transfection analyses with compound artificial promoters, the INR alone was not as active as the TATA box alone (see, for example, reference 12), and it is probable that this is true for MLP in its natural context as well. This interpretation is supported by the observations with INR mutations coupled to those in upstream activating sequences. Thus, the double-mutant virus USF0::start-1 is transcriptionally deficient but viable (Fig. 2 and 4), whereas USF0::TATA30 is inviable (35). The comparison between the behaviors of the CCCAT::TATA27 and CCCAT::INR-5 mutant viruses may be particularly revealing, because the TATAGAA sequence in TATA27 should bind TBP at some 25% efficiency (6), and it seems likely that the INR-5 and start-1 mutations would have at least some binding deficiency. Yet the CCCAT::TATA27 mutant virus has a transcriptional deficiency of some 20-fold (35), while the CCCAT::INR-5 is normal (Fig. 4).

The data presented in Fig. 2 and 4 also support the idea that the activity of the activating factor USF, which binds to the UPE, is exerted at least in part via the protein that binds to the INR, because the mutant virus USF0::start-1 is transcriptionally deficient. However, the interaction between USF and the INR-binding protein, which could be direct or indirect, is not sufficient by itself to allow maximal transcription from the MLP because CCCAT::TATA27 has a severe transcriptional deficiency (35). In the latter virus, binding of CP1 (the factor that binds to the inverted CAAT box [9]) and binding of TBP to the TATA box are both expected to be lowered, and transcription would then be more dependent on the hypothetical interaction between USF and the INR-binding protein. Finally, there is no evidence that CP1 interacts with the INR-binding protein, because as mentioned above, CCCAT::INR-5 has no phenotype. Taking all of the evidence of the present and previous studies (34, 35) together, we believe it likely that the interaction between USF and TBP is the most crucial of any of the potential interactions between the activating and basal factors for maximal expression from the MLP. Biochemical data suggest that this interaction is mediated via one of the TBP-associated factors rather than TBP itself (26), but the identity of the TAF is not yet known.

Are the genetic data discussed above compatible with two recent models for the function of the INR? In one model, a sequence-specific polypeptide, TFII-I, which has immunological similarity to USF (37), acts as an alternative nucleating site for the assembly of the preinitiation complex (30, 36) and recruits TFIID to TATA-less promoters by protein-protein interaction. This model predicts that mutation in either the TATA box or the INR alone would have minimal effects on MLP function, but double mutations would be severely deficient, as is the case for mutants TATA30::start-1 and TATA30::INR-5. The model can be modified readily to take account of the relative contributions of the two elements to basal transcription. If, in addition, USF interacts with TFII-I, this could explain the phenotype of USF0::start-1. An alternative model is based on the observation that one of the TAFs, namely, $TAF_{II}150$, can bind specifically to the INR of the MLP in vitro (50). It has been known for many years that TFIID makes a much more extended footprint on the MLP (39) than does purified TBP, and more recent evidence showed that *Drosophila* TBP and $TAF_{II}150$ ($dTAF_{II}150$) together make a similar extended footprint on the MLP (50). As with TFII-I, $dTAF_{II}150$ might be capable of recruiting TBP to a TATA-less promoter by protein-protein interactions. In vitro transcription assays with the MLP, TBP, $dTAF_{II}150$ and $dTAF_{II}250$, showed that the TAFs can discriminate between promoters with or without an INR (49) and may even decrease the stability of the preinitiation complex in promoters lacking an INR. This last observation is not consistent with the genetic data, but it remains to be seen if this is a consequence of the relatively minor mutational changes made in the genetic studies compared with the deletions used in the biochemical assays (49). Further evidence that $TAF_{II}150$ plays a role in INR-mediated transcription initiation comes from a recent report showing that a fraction termed CIF was necessary for TFIID to function in vitro at the INR of the terminal deoxynucleotidyltransferase gene (24). Both functional and immunological analyses suggested that one of the components of CIF is likely to be the human homolog of $dTAF_{II}150$. As a final comment on function, $dTAF_{II}150$ has also been shown to be the coactivator for a number of activating factors (8), and it is tempting to speculate that the phenotype of USF0::start-1 reflects the inability of USF to interact with this TAF at the double-mutant MLP. As mentioned above, although USF activates via TFIID rather than TBP (26), the identity of the TAF through which this activation is accomplished is not known.

The MLP and the IVa2 promoters are the only adenovirus promoters for which there is experimental evidence of an INR (5, 6, 37, 43). However, by inspection of the sequences at the start sites of the other viral promoters (41), it is clear that several have close matches to the loose consensus PyPyAN(A/T)PyPy, with the E1A and E4 promoters having an exact match and E1B and pIX having one mismatch, as does the known INR-containing IVa2 promoter. It would be of some interest to know if these other viral promoters contain a functional initiator, not only to gain a better understanding of the structure of viral promoters in general but also because they are sometimes used experimentally to investigate the mechanisms of transcriptional activation. As pointed out by Emami et al. (16), the E4 promoter was used by others (7) as an example of a promoter that lacks an INR, and based in part on that assumption, it was claimed that Sp1 activates via the TATA box rather than the INR. This conclusion is almost certainly wrong (12, 16). The studies on core promoter selectivity and the role of $TAF_{II}150$ (49) also used the E1B and E4 promoters as erstwhile INR-lacking promoters. Clearly it would be valuable to have biological evidence of the presence or absence of INR function at these other viral promoters.

Both of the models for INR function discussed above propose that the protein that binds to the INR makes contacts with TBP. Therefore, the next step in the genetic analysis is to see if the spacing of the INR and the TATA box is critical to their joint function in the viral genome. Such experiments have been performed with the IVa2 promoter in vitro, where insertions reduced both the quantity and the accuracy of initiation (5). However, it is worthwhile to examine this question in vivo, not only because the IVa2 promoter is unusual in having the TATA box located in inverted orientation downstream of the start site (6, 23) but also because our previous experience (34, 35) has demonstrated that results in vitro are frequently not reproduced in vivo. Indeed, we have shown recently that insertions of 3 and 6 bp between the TATA box and the INR have no effects on the frequency of initiation (29a). It remains to be seen if the potential disruption to the interaction between

TBP and the INR-binding protein is of greater transcriptional consequence when the TATA box has been mutated, and the presence of TBP at the promoter then depends on protein recruitment rather than intrinsic DNA binding.

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