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Transcription from the adenovirus major late promoter uses redundant activating elements

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The adenovirus major late promoter (MLP) has been analyzed by constructing recombinant viral genomes containing mutations in possible promoter elements. Single base pair changes in the TATA box had no effect on viral replication, and MLP expression, as measured by the accumulation of late mRNAs, was at wild type levels. However, a double mutation in the TATA box reduced viral replication and MLP expression, demonstrating that the TATA box is important, although not essential, for maximal activity in virus. Primer extension analysis showed that the mRNAs were initiated at the correct position. A mutation in the CAAT box was viable, and had only minor effects on MLP expression. However, this mutation when coupled to a single mutation in the TATA box, severely reduced viral replication and expression from the MLP. Similarly, a viable mutation in the UPE, shown previously to abolish binding of USF, coupled to a single mutation in the TATA box was lethal. These results suggest that both USF and the CAAT box binding factor CP1 can interact with TFIID to effect activation, and thus that the mechanism of activation is functionally redundant.

Key words: adenovirus major late promoter/promoters/ transcription regulation

Introduction

The molecular mechanisms by which transcription is regulated at mammalian pol II promoters are proving to be extremely diverse (reviewed in Berk and Schmidt, 1990). Nevertheless, it is generally believed that a distinction can be drawn between elements of promoter structure and transcription factors that are required for basal activity, and those which activate transcription from the basal level. The interactions between the basal machinery and the activating proteins, and the specific DNA–protein and protein–protein interactions involved in activation, remain to be elucidated.

The most common genetic methods for studying promoter structure have been reductionist. Point mutations, linker scanning insertions and deletions in known or suspected promoter elements have been linked to experimentally convenient reporter sequences. These constructs are studied in *in vitro* transcription assays, plasmid-borne transfection assays, or by placement ectopically in a viral or cellular chromosome. Each assay yields important information on the structure and function of the promoter. However, the effects of the native genomic context and the correct temporal and spatial transcription program, both of which will influence a promoter's regulated expression, will not be apparent. The importance of the correct biological context is addressed specifically in this paper, in which we examine mutations in one of the best studied mammalian promoters, the adenovirus major late promoter (MLP), in its correct genomic location.

Previous analyses of the MLP have shown it to be functionally simple, requiring a TATA box for basal transcription activity and an upstream element, UPE, for maximal activation (Hu and Manley, 1981; Hen *et al.*, 1982; Lewis and Manley, 1985; Logan and Shenk, 1986; Lee *et al.*, 1988), reviewed in Berk (1986). The genetic evidence is strongly supported by biochemical results which showed an interaction between the two protein factors, TFIID and USF, which bind to the TATA box and the UPE respectively (Sawadogo and Roeder, 1985). These results have been repeated with purified factors in a chromatin assembly assay (Meisterernst *et al.*, 1990). Thus activation at the MLP can be achieved through recruitment of the general transcription factor TFIID by the activating factor USF. However, evidence from the viral life cycle has shown that there are alternative mechanisms of activation. The MLP is subject to activation by the transactivating protein encoded by the viral E1a gene (Nevins, 1981; Lewis and Manley, 1985), and DNA replication also is required for maximal levels of expression (Thomas and Mathews, 1980). Recently we have shown that a virus lacking a functional UPE site replicates normally (Reach *et al.*, 1990), despite evidence from *in vitro* and transfection assays that this site is necessary and sufficient for maximal activation. In addition, we suggested that an inverted CAAT box, located upstream of the UPE and thought to play no part in the activation of the MLP, could compensate for the absence of USF binding (Reach *et al.*, 1990). Taken together, these *in vivo* results show that the MLP is subject to many levels of control. This prompted us to examine by mutagenesis all of the known or suspected promoter elements of the MLP, to determine their roles in normal transcriptional control.

In this paper, we demonstrate that the TATA box is important but not essential for accurate transcription from the MLP, and that the inverted CAAT box is important for normal activity through an interaction with the TATA box. In addition, one of the downstream elements which are thought to play a role in the activation of the MLP following DNA replication (Jansen-Durr *et al.*, 1989), is dispensable.

Results

A genetic analysis of the elements of the MLP required in virus

The elements of the MLP and their binding factors are shown in Figure 1. The basal elements are a TATA box that binds TFIID (Sawadogo and Roeder, 1985; Nakajima *et al.*, 1988), and an initiator element (Concino *et al.*, 1984; Smale and Baltimore, 1989; Garfinkel *et al.*, 1990; Smale *et*

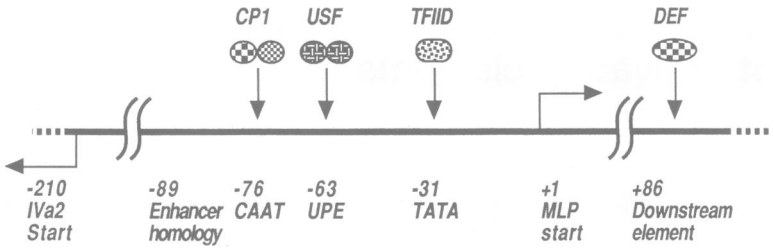


Fig. 1. Known or suspected elements of the MLP. The elements are shown with their cognate binding factors. The region is located at ~16.8 m.u. from the left hand end of the viral genome with the MLP start site at bp 6030. The divergent IVa2 promoter is also shown. The region lies within the coding sequences of the essential DNA polymerase gene, which is encoded on the opposite strand.

al., 1990) that includes the transcriptional start site. The activating elements are the UPE that binds USF (Sawadogo and Roeder, 1985; Chodosh *et al.*, 1986; Lennard and Egly, 1987; Gregor *et al.*, 1990), an inverted CAAT site that binds the transcription factor CP1 (Chodosh *et al.*, 1988), and a downstream element that binds a factor known as DEF (Jansen-Durr *et al.*, 1989). There is also a sequence showing homology (Berk, 1986) to an E1a enhancer element, whose role has never been examined before.

Mutations in the various elements were made by oligonucleotide-directed mutagenesis (see Table I and Materials and methods). The effect of each mutation was tested by the viral MLP replacement assay described previously (Reach *et al.*, 1990). Briefly, the plasmid containing the mutation, located in a left terminal viral sequence, was co-transfected with an overlapping right terminal fragment of the genome into human A549 cells. Recombination in the overlapping interval will result in a full length genome. If the mutated MLP is as transcriptionally active as the wild type promoter, the genome will give rise to viruses inducing normal plaque morphology, or, if transcription is lowered, the size and number of plaques will be reduced. If no plaques are produced, it is presumed that the mutation is lethal, because of insufficient expression from the MLP.

Table I. Sequences of the mutated elements

Element	WT sequence and coordinates	Mutant sequence	Virus name
	−31 −25		
TATA	TATAAAA	T**G**TAAAA	TATA30
		TATA**G**AA	TATA27
		T**G**TA**G**AA	TATA0
	−3 +6		
Initiator	CTCACTCTC	CTC**G**CTCTC	Start-1
	−90 −80		
E1a enhancer homology	AAGGAAGGTGA	**G**AG**A**AA**T**GTGA	E1a−enh
	−80 −76		
CCAAT box	TAACC	TA**C**CC	CCCAT
		TA**G**CC	CCGAT
	+85 +95		
Downstream element	ATTGTCAGTTT	**G**TT**A**TC**C**GT**G**T	DEF

The analysis of mutations in basal elements

The adenovirus MLP has two elements that play a role in basal levels of transcription and in accurate initiation, the TATA box and an initiator element. Although these two elements have been examined *in vitro* and in transient transfection assays, their role in virus production has never been determined. A canonical TATA sequence is located at the appropriate distance from the transcriptional start site (see Figure 1 and Table I). Previous evidence has shown that it plays an important role in basal levels of transcription, and also that it is the site of activation by both E1a (Leong *et al.*, 1988) and USF (Sawadogo and Roeder, 1985). Wobbe and Struhl (1990) made a complete set of all possible point mutations in the TATA box of the MLP. Most point mutations resulted in decreased MLP activity, ranging from 72% to <1% of the activity of the intact TATA box, in an *in vitro* transcription system, as well as in an *in vivo* assay. The full range of point mutations cannot be tested in virus, because of constraints caused by the DNA polymerase encoded on the opposite strand. Thus, only two point mutations could be made and tested, but, fortunately, these mutations, (TGTAAA) and (TATAGAA) at −30 and −27, were those in which promoter function was reduced to 3 and 5% respectively *in vitro*. Strikingly, both mutations gave rise to viable viruses. Neither point mutation had an effect on viral propagation, as illustrated by one step growth curves for TATA30 and TATA27 (Figure 2A), which demonstrates that the eclipse period and growth rates of the two mutants are identical to wild type. Furthermore, the mutations did not reduce MLP expression, as demonstrated by RNase protection assays (data not shown). Primer extension analysis showed that the transcription start site was unaffected by the point mutations (see Figure 4, TATA27). These results show that the sequence requirements for the TATA box in virus are less stringent than those observed in other assays.

One interpretation of the results with the single point mutations is that the residual binding of TFIID is sufficient for maximal expression from the MLP. To try to eliminate binding, a promoter containing two nucleotide changes in the TATA sequence at −30 and −27 (TGTAGAA) was constructed. This mutated TATA box does not resemble any known TFIID binding site and does not bind TFIID *in vitro* (D.Reinberg, personal communication). Despite the absence of an intact TATA box, viable virus (termed TATA0) was obtained. However, the plaque size was reduced, the growth rate was slower, and the eclipse period was protracted compared with the wild type (Figure 2A). Promoter expression, as measured by RNase protection assays, was decreased by 10- to 20-fold compared with the wild type, at 16 h post-infection (Figure 3A). The level of E1b mRNA was elevated at these times, but was equivalent at 6 h post-infection (data not shown), demonstrating that the

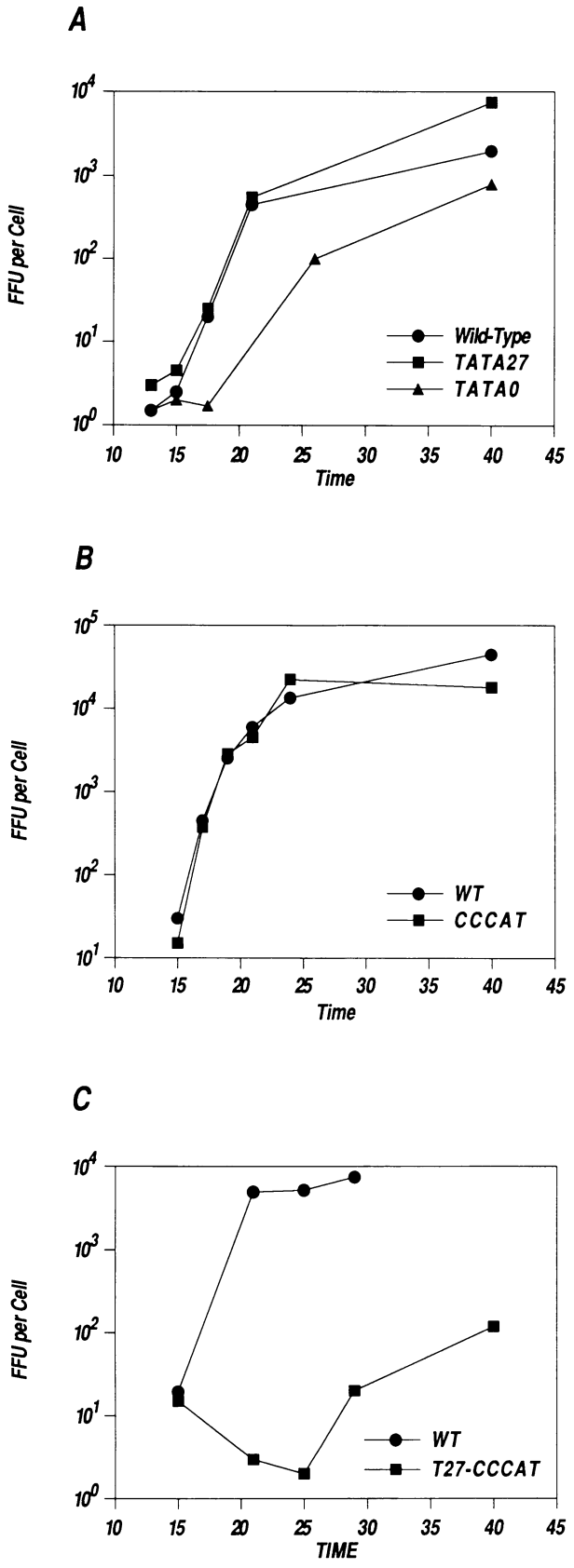


Fig. 2. Viral replication cycles. A549 cells in monolayer culture were infected at an m.o.i. of 10 p.f.u. per cell, and individual dishes were taken at intervals. Virus was titrated by fluorescent focus assay. The mutant viruses are as follows: (**A**) TATA27 and TATA0; (**B**) CCCAT; (**C**) TATA27–CCCAT. Wild type infections were conducted and titrated in parallel.

input of genomes and the early stages of transcription were similar in the two infections. The marked reduction in MLP expression is consistent with the lowered replicative ability. Primer extension analysis demonstrated that transcription initiation from the MLP was accurate (Figure 4, TATA0).

Together, these results suggest that the TATA box is important, but not essential, to promoter function and that other elements can not only activate transcription from the mutated promoter, but also accurately position the start site. In addition, these results also suggest that the other basal element, the initiator, is not sufficient to allow maximal transcription from the MLP in the absence of functional TFIID binding.

Previous studies *in vitro* and in transient assays have demonstrated that mutations in the initiator element at +1 decreased promoter expression up to 10-fold (Concino *et al.*, 1984; Lee *et al.*, 1988; Smale *et al.*, 1990). A virus containing an A to G transition at +1 was constructed, and replicated normally (data not shown). Although this could be taken to imply that the transition mutation had no effect upon the binding of factors necessary for initiator function, we have recently constructed a virus containing the +1 transition and the −30 mutation in the TATA box. This mutant virus replicates extremely slowly (data not shown), suggesting that the mutation at +1 does indeed affect binding.

Activating elements

Previous studies have demonstrated the importance of the UPE to MLP activation but, as we have shown previously (Reach *et al.*, 1990), its role may be substituted by the CAAT box. The functions of the CAAT box and two other possible activating elements have now been examined.

CAAT box. Although most studies have shown that the MLP does not require sequences upstream of the UPE, an inverted CCAAT sequence is located upstream of the UPE (Figure 1). It has been shown to bind a heterodimeric cellular factor, CP1, *in vitro* (Chodosh *et al.*, 1988a,b).

Mutations in the central A, changing the sequence from CCAAT to CCCAT or CCGAT did not affect viral growth, as illustrated in the growth curve for the virus CCCAT (Figure 2B). However, the CCCAT mutation decreased MLP expression by 2- to 5-fold at 16 and 24 h post-infection, as demonstrated by RNase protection assay (Figure 3B and C). Because the reduction in MLP expression had no apparent effect on viral replication, the implication is that other steps in the biosynthesis and assembly of virus are rate limiting.

A downstream binding site. Downstream of the start site is a region that activates MLP expression at late times (Mansour *et al.*, 1986; Leong *et al.*, 1990). Nuclear factor(s), detected only in extracts obtained from cells in the late phase of infection, bind to sequences downstream of the MLP and activate transcription *in vitro* (Jansen-Durr *et al.*, 1989; Leong *et al.*, 1990).

A four nucleotide mutation (Table I) was created in the downstream element defined by Jansen-Durr *et al.* (1989). Two of the mutations are transitions in nucleotides which, when methylated, interfere with complex formation *in vitro*; one is a transversion at a central position and the other is a transition at position +85 immediately adjacent to the

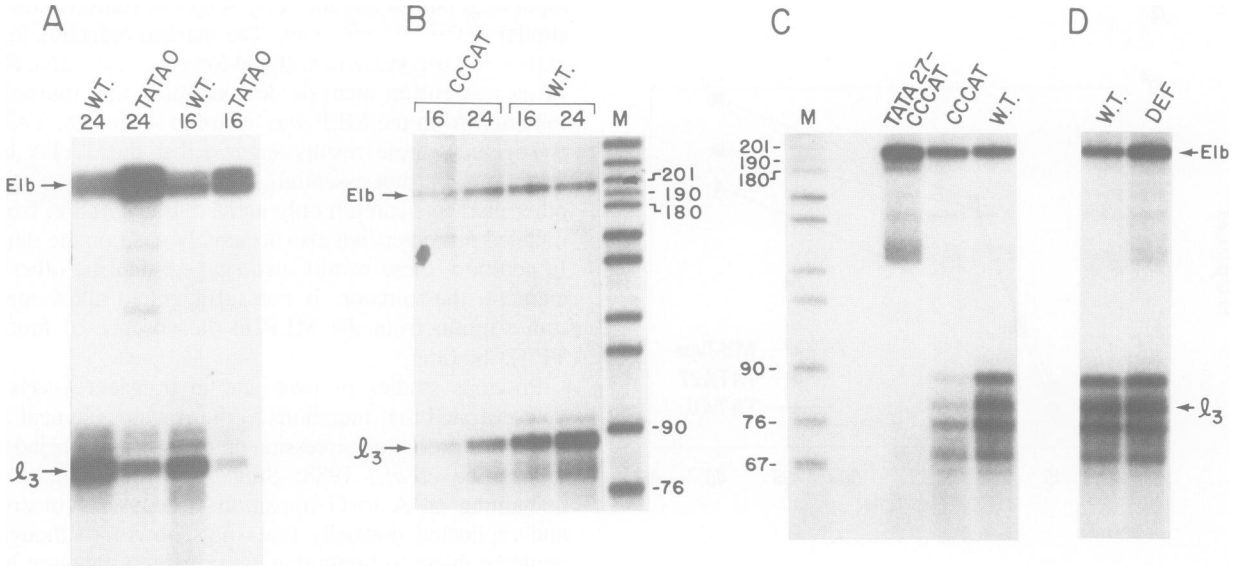


Fig. 3. Steady state viral mRNA levels. Cells infected with wild type or mutant viruses were harvested at 16 and 24 h post-infection (panels **A** and **B**) or at 24 h only (**C** and **D**). Sequences protected from RNase T2 digestion by the riboprobes are specific for E1b and late mRNA leader 3 (I_3) and are 195 and 89 nucleotides long respectively (Reach *et al.*, 1990). The latter is often observed as a set of bands of different intensity. The hybridization and T2 digestion were performed on the samples of RNA with a mixture of the two riboprobes. The protected mRNAs are from the following infections: (**A**) TATA0 and wild type; (**B**) CCCAT and wild type; (**C**) TATA27−CCCAT, CCCAT and wild type; and (**D**) DEF and wild type. Markers (M) are end-labeled fragments of *Msp*I-digested pBR322. Quantitation of the bands was by densitometry scanning.

proposed binding site. The mutated sequence had no effect on viral growth and MLP expression was not impaired at 24 h post-infection as compared with that of the wild type (Figure 3D).

The interaction of promoter elements

Our results show that mutations in any single activating element do not diminish the replicative ability of the virus, and, at best, have minor effects on MLP expression, observations that may be explained by functional redundancy of the elements. On the other hand, the results with the TATA0 virus strongly support the idea that the TATA element is the most important in basal and/or activated expression from the MLP.

While the importance of any one element can be revealed by mutagenesis, the interactions between elements must be studied with combinations of single mutations. The results with such double mutations may reveal interactions between the basal transcriptional machinery and the activating factors, and the relative importance of individual activating promoter elements in the control of MLP expression.

TATA box−UPE. The four base mutation in the UPE decreased MLP activity 2- to 5-fold 9 h post-infection, while at 20 h expression was close to normal (Reach *et al.*, 1990). The viral replication cycle was indistinguishable from that of the wild type. The viral mutants TATA30 and TATA27 which contain single point mutations in the TATA box display normal growth and RNA expression (Figure 2A and data not shown). However, in repeated attempts at reconstruction, the double UPE−TATA mutations could not be incorporated into virus, strongly suggesting that these double mutations are lethal to the virus. To confirm this conclusion, an alternative strategy was performed using marker rescue with the plasmid containing the TGTAAA and UPE mutations (Figure 5). In this technique, virus

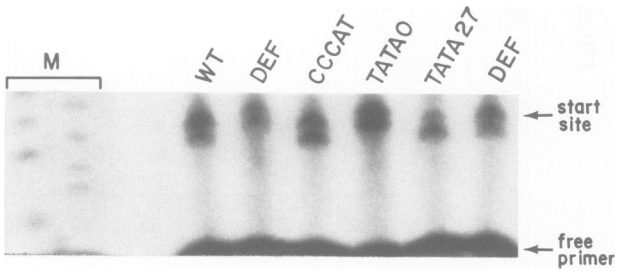
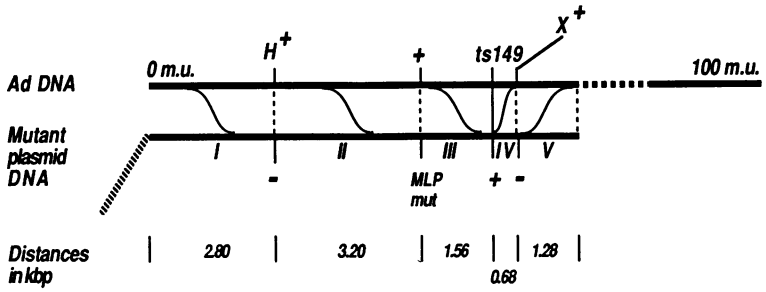


Fig. 4. Primer extension analysis. Steady state mRNA from cells infected with the virus shown at the top of each lane was isolated at 24 h post-infection and hybridized to a primer 25 nucleotides long. Reverse transcription to the start site 11 nucleotides upstream should produce an extension product of 36 nucleotides, as confirmed by the size markers (M).

is recovered and the inheritance of restriction site polymorphisms and the mutations of interest are scored. Previous work has shown that the inheritance of markers of known location can be predicted with precision (Volkert *et al.*, 1989). Restriction mapping and sequence analysis of individual ts^+ viral DNAs showed that neither the restriction site marker upstream of the mutant promoter, nor the mutated bases themselves were inherited by the offspring. The absence of these sites is significant at the $P < 0.01$ level and strongly supports the idea that the double mutation in the UPE and TATA box is lethal.

Taken together, these observations suggest that the sequences TGTAAAA and TATAGAA have lower affinities for TFIID in the virus genome, and require an intact UPE, and the binding of USF, to stabilize TFIID binding to the mutant TATA box. These results are in agreement with those which showed that the USF interacts cooperatively with TFIID, and that this interaction stabilizes the binding of both factors (Sawadogo and Roeder, 1985).



Crossovers in interval	I	II	III	IV	V
Observed:	0/11	0/8	8/8	3/11	8/11
Expected:	4/11	5/8 *	3/8 *	4/11	7/11

Fig. 5. Testing of lethality by marker rescue. To produce a ts^+ virus, recombination (∿) must occur in intervals I, II or III and intervals IV or V. Eleven isolates were examined by restriction digestion, and the MLP region was cloned and sequenced from eight of them. Symbols: $H^{+/-}$ $X^{+/-}$, the presence or absence of the *Hin*dIII site at bp 2798 and of the *Xho*I site at bp 8244 respectively; in each case, the absence of the site is caused by a single base pair change which has no phenotypic consequences; MLPmut, the double mutation containing the 4 bp change to the UPE and the TGTAAAA mutation in the TATA box. The expected frequencies of crossovers in the intervals were calculated on the basis of the physical distances between markers, shown in kbp. * The probability of the observed versus the expected values ($P < 0.01$) was calculated by the G test with Yates correction (Sokal and Rohlf, 1969). In a parallel experiment with a phenotypically silent six base *Eco*RI insertion mutation located 100 nucleotides downstream of MLPmut, two or three recombinants had inherited the *Eco*RI site.

TATA box−CAAT box. The mutations TATAGAA and CCCAT individually had no effect upon viral replication (Figure 2A and B), and the latter had only minor effects upon MLP activity (Figure 3B). To our surprise, the double mutation gave rise to a virus, TATA27−CCCAT, that had a severely defective growth rate. Plaque size was reduced, the final yield of virus in the one step growth curve was decreased by two orders of magnitude, and the eclipse period was extended by several hours compared with the wild type infection (Figure 2C). Activity of the mutant MLP was reduced some 25-fold compared with that of the wild type (Figure 3C). This decrease is the greatest observed with all of the mutations that could be incorporated into virus.

These results strongly suggest that in the native wild type genome, TFIID can interact with CP1 to effect activated transcription from the MLP. Furthermore, USF binding to the TATA27−CCCAT genome, in the absence of strong binding of TFIID and CP1, is insufficient to give high levels of expression from the MLP.

TATA box−downstream element. The double mutation containing TGTAAAA and the four base mutation in the downstream element was created. As mentioned above, neither single mutation affects viral replication and nor does the double mutation (data not shown). This result suggests that DEF, unlike the other activating factors, does not interact with TFIID.

Combinations of mutations in activating elements. As mentioned above, our previous results suggested that the UPE and CAAT box are functionally redundant (Reach *et al.*, 1990). Similarly, the downstream element may be redundant with one or other of the upstream elements. However, double mutations in the downstream element and with either the UPE or the CAAT box gave rise to virus with normal replication cycles and with MLP expression similar to that of the single parents (data not shown).

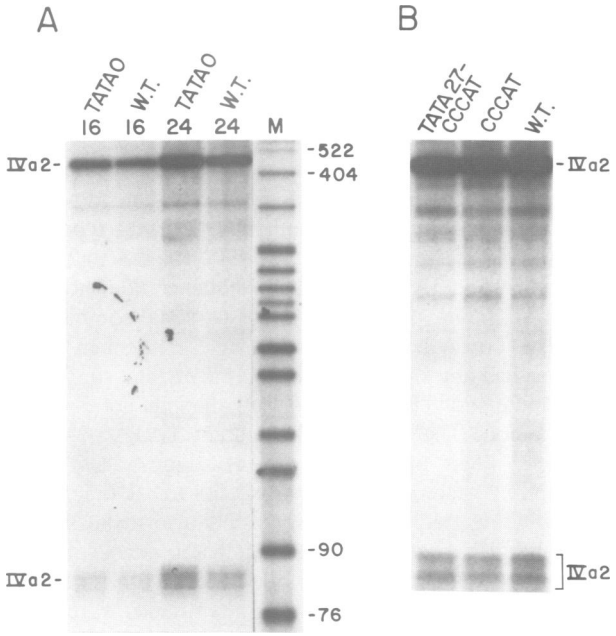


Fig. 6. Steady state levels of IVa2 mRNA. (**A**) RNA isolated from TATA0 and wild type infected cells at 16 and 24 h post-infection, hybridized to a riboprobe specific for the divergent IVa2 promoter forms two protected fragments 441 and ~90 nucleotides long. (**B**) Protected TATA27−CCCAT, CCCAT and wild type mRNA harvested at 24 h post-infection. Quantitation of the bands was by densitometry scanning. Markers (M) are as described in Figure 3.

The divergent IVa2 promoter

The MLP is adjacent to a divergent promoter for the IVa2 gene, whose start site is separated from that of the major late transcription unit by 210 nucleotides (Figure 1). IVa2 probably encodes an essential scaffolding protein, and is only active after DNA replication (Crossland and Raskas, 1983). The mechanism by which the divergent IVa2 promoter is

regulated, both in activity and in temporal control, is unclear. However, analysis of the single and double mutant viruses indicated that mutations in any of the activating elements, or in the TATA box, have no detectable effects on IVa2 activity. Representative experiments for TATA0, CCCAT and TATA27−CCCAT are shown in Figure 6. These results suggest that the divergent promoters are under separate control in virus, and that simple sharing of factors, or competition for transcription initiation complexes does not contribute to their relative activities.

Discussion

The aim of the genetic analysis described in this paper was to examine the roles of the known or suspected promoter elements in the functioning of the adenovirus MLP in virus. The results demonstrate that the functioning of the promoter is different from that predicted from previous *in vitro* transcription or transient transfection assays.

Basal elements

The adenovirus MLP contains a consensus TATAAA sequence at the appropriate distance upstream of the start site. As with other pol II promoters, it is believed that this is an essential element of the MLP (Concino *et al.*, 1984; Wobbe and Struhl, 1990). Recent evidence suggests that another element, the initiator, centered on the start site, may be functionally redundant with the TATA box in supporting correct and quantitative initiation of transcription (Smale and Baltimore, 1989; Smale *et al.*, 1990). To test the requirements for the basal elements, a series of mutations was reconstructed into virus. Single point mutations in the TATA box had no phenotypic effects by themselves (Figure 2A), but a double mutation, TGTAGAA, yielded a virus which replicated much less well than the wild type, and expression from the MLP was reduced some 10- to 20-fold (Figures 2A and 3A). This mutant sequence does not bind purified TFIID *in vitro* (D.Reinberg, personal communication). The TATA box is the only single element in which mutations had any significant phenotypic effects, suggesting that the binding of TFIID is important, although not essential, to expression. The fact that the mutant TATA0 MLP is expressed, albeit to a lesser extent, suggests that other transcriptional factors can substitute for the deficiency in TFIID binding. However, these results also make it clear that an alternative basal element, the initiator, is not sufficient to substitute quantitatively for the absence of functional TFIID binding in virus. On the other hand, a recently constructed virus containing single point mutations in both basal elements has a significant deficiency in virus replication (data not shown), despite the lack of any phenotypic consequences of either mutation alone. This suggests that the two basal elements may be partially redundant, and agrees with evidence *in vitro* which showed that mutations in both of the basal elements together led to a marked reduction in transcription (Concino *et al.*, 1984).

Interactions between the TATA box and the activating elements

As mentioned above, single point mutations in the TATA box have no phenotype (Figure 2A), even though each mutation has been shown to decrease activity of the MLP *in vitro* to 5% or less of that of the wild type (Wobbe and Struhl, 1990) and, in the case of TATAGAA, to bind TFIID less efficiently (Carcamo *et al.*, 1990). The lack of effect in virus can be explained if the lowered affinity is compensated, directly or indirectly by the action of the other transcription factors. This explanation is supported by the evidence that double mutations containing single point mutations in the TATA box and mutations in either the UPE or the CAAT box have severely deficient phenotypes. The lethality of coupled mutations in UPE and the TATA box, confirmed in two different types of reconstruction assays (Figure 5), can be interpreted as evidence for an interaction between USF and TFIID. This is in agreement with biochemical observations suggesting a direct interaction between the two proteins (Sawadogo and Roeder, 1985; Meisterernst *et al.*, 1990), and may reveal one of the ways in which the MLP is activated. The lethality of the double UPE−TATA mutation also suggests that the binding of CP1 to the intact CAAT box provides insufficient expression from the MLP to allow the recovery of virus. More surprising was the observation that a double mutation containing changes in the CAAT and TATA elements conferred a seriously deficient transcriptional phenotype upon the virus (Figure 3C). One interpretation of this observation is that transcription factor CP1 is capable, like USF, of protein−protein interactions with TFIID. This interpretation differs from a previous conclusion that there are two families of upstream factors, one of which can interact with TFIID and the initiation complex, while the other, which includes CAAT binding factors, cannot (Taylor and Kingston, 1990). However, it is also possible that the mechanism of interaction is different and that the apparent interaction between the CAAT and TATA boxes is indirect. Recent evidence from other promoters suggests that the interaction between TFIID and some activators may be indirect (Kambadur *et al.*, 1990; Pugh and Tjian, 1990). The availability of the purified CP1 transcription factor would allow the direct interaction hypothesis to be tested. However, regardless of the precise nature of the interaction between the two, the results with the TATA27−CCCAT mutant virus support our previous suggestion that the UPE and CAAT box are functionally redundant (Reach *et al.*, 1990).

Other genetic elements

This genetic survey included an analysis of other suspected promoter elements, including an upstream sequence showing homology to an E1a enhancer element (Berk, 1986). However, a three base mutation (Table I), either singly or in combination with mutations in the TATA element or the UPE had no phenotypic consequences (data not shown). It is unclear if this has any role in virus or indeed if it is a genuine promoter element. More surprisingly, no phenotypic consequences of the four base mutation in the recently described downstream element were observed either singly or in combination. However, the downstream region itself may be redundant, containing multiple elements (Jansen-Durr *et al.*, 1989; Leong *et al.*, 1990) that might compensate for the loss of function of the four base mutation. It remains to be determined if coupled mutations in multiple downstream elements have any phenotypic consequences in virus.

The divergent IVa2 promoter

None of the mutations had any effect upon the activity of the IVa2 promoter. Thus, contrary to results from *in vitro*

and transient transfection assays (Natarajan *et al.*, 1985; Mermelstein *et al.*, 1989), we can conclude that the divergent promoter does not share elements with the MLP and that competition for transcription factors is not involved in the divergent regulation. This is in line with more recent evidence that IVa2 may possess its own set of regulatory elements, including its own TFIID binding site and initiator element (Carcamo *et al.*, 1990), and a site which binds USF with an affinity lower than that of the UPE (Moncollin *et al.*, 1990).

Why do the results with MLP mutations in virus differ from those using other assays?

At the outset of this investigation it was not clear if the elements of the MLP identified previously would be important to the activity of the promoter located in the specific nucleoprotein structure of the viral genome, and subject to the correct temporal and perhaps spatial regulation. The results in this paper demonstrate discrepancies between the results obtained in virus and those from previous studies. The TATA box is not essential for promoter activity, but nor is its function quantitatively compensated by the initiator element. The CAAT element is functionally redundant with the UPE, and, furthermore, CP1 may interact with TFIID. Transcription from the divergent IVa2 promoter is not affected in mutations that have severely deficient transcription from the MLP. The reasons for these differences are unknown, but could reflect the effects of the viral E1a transactivating protein, of distant enhancer elements, or of changes in the activities of rate limiting components in transcription initiation. However, whatever the causes of these differences, it is clear that the viral reconstruction assay adds an important biological component to the analysis of promoter structure and function.

Materials and methods

Oligonucleotide-directed mutagenesis

A 453 bp adenovirus DNA sequence extending from a *Xho*I site at bp 5778 to a *Hin*dIII site at bp 6231 was cloned into the same sites in an M13mp18 vector (Yanisch-Perron *et al.*, 1985) containing the polylinker from pIC7 (Marsh *et al.*, 1984). Mutagenesis was performed by the techniques of Kunkel (1985) as described in detail in Reach *et al.* (1990). The mutant oligonucleotides were obtained from the Columbia Cancer Center DNA facility and from Genosys, The Woodlands, TX. M13 plaques were screened for those containing the desired mutations, by sequencing the viral DNA with the dideoxy method (Sanger *et al.*, 1977).

The testing of MLP mutations by overlap recombination

After mutagenesis, the *Xho*I–*Hin*dIII MLP fragment was excised from the M13 vector RF DNA and cloned into the replacement vector pMR2, which contains adenovirus sequences extending from the left terminus to bp 9523 (Reach *et al.*, 1990). The viability of a particular mutant MLP was tested by the methods described previously (Reach *et al.*, 1990). DNA preparations of the mutation-containing plasmid were restricted with *Eco*RI, which cleaves at the left terminus. After inactivation of the restriction enzyme, the fragment was co-transfected (Graham and Van der Eb, 1973), on A549 human lung carcinoma cells (Giard *et al.*, 1991), with a right terminal adenovirus genomic fragment extending from the *Xho*I site at bp 8244 from the phenotypically wild type virus LLX1 (Brunet *et al.*, 1987). Recombination in the overlapping sequence between bp 8244 and bp 9523 will yield a full-length genome, and, if the mutation does not confer a lethal phenotype, will give rise to plaques on the transfection plates. The presence of the mutations in the resulting viruses was confirmed by cloning the *Xho*I–*Hin*dIII MLP-containing fragment into a pSP64-derived plasmid, followed by double stranded sequencing using the method of Zagursky *et al.* (1985). Mutant viruses were plaque purified once before further analysis.

Testing of potentially lethal mutations by marker rescue

The methods for marker rescue and the analysis of recombinant viruses have been described in detail (Volkert *et al.*, 1989). Briefly, A549 cells in 35 mm culture dishes were co-transfected with 100 ng of DNA–protein complex from the temperature-sensitive mutant H5*ts*149, which contains a C to T transition at bp 7563 in the DNA polymerase (Roovers *et al.*, 1990), and with 300 ng of the pMR2 derivative containing the mutations of interest. After incubation of the transfected plates at the permissive temperature for 3 days, yields were harvested, and wild type viruses were selected by plaque assay on A549 cells at the restrictive temperature. Individual ts^+ virus isolates were examined by restriction of intracellular viral DNA with *Xho*I and *Hin*dIII and the MLP region was cloned and sequenced from several isolates. Previous work has shown that the inheritance of restriction sites and other markers can be predicted with accuracy if their physical positions are known (Volkert *et al.*, 1989).

Viral replication

Growth curves were performed on A549 cells in 35 mm culture dishes. Cells were grown to confluency in DME + 10% supplemental calf serum (Hyclone). Infections were performed by removing the medium and adding 0.2 ml of virus at a multiplicity of infection (m.o.i.) of 10 plaque-forming units (p.f.u.) per cell and incubating at 37°C for 1 h, with periodic shaking. After adsorption, the plates were overlaid with infecting fluid (Lawrence and Ginsberg, 1967). The infected cells were harvested at intervals by freezing individual dishes, and virus was liberated by repeated freezing and thawing. Titration was performed on A549 cells by fluorescent focus assay (Philipson, 1961).

RNase protection assays

Riboprobes specific for E1b, IVa2 and late leader 3 were made by incubating the appropriate linearized template with SP6 RNA polymerase under standard conditions (Melton *et al.*, 1984). Amounts of RNA corresponding to 1×10^6 c.p.m. of the riboprobe preparation were used in the subsequent hybridization reactions. Cytoplasmic RNA was isolated at the indicated times post-infection from confluent monolayers of A549 cells in 10 cm dishes. Approximately 2×10^7 cells were pelleted, washed once with ice-cold PBS without magnesium, resuspended in 1 ml of PBS and transferred to a microcentrifuge tube. The solution was extracted once with RNAzol, Cinna Biotecx, Friendswood, TX, and the RNA was precipitated with ethanol. Hybridizations between the cytoplasmic RNA and the labeled riboprobe were performed in 75% formamide, 0.4 M NaCl, 0.1 mM EDTA for 18 h at 62°C. Protection was performed with RNase T2 at a range of inputs of cytoplasmic RNA, and at late times it was found that 0.1 μg of RNA was within the linear range. After 2 h digestion, the mix was phenol extracted and the subsequent ethanol precipitate was electrophoresed on a 6% denaturing acrylamide gel. The sizes of the expected protected sequences are given in the figure legends.

Primer extension analysis of cytoplasmic viral RNA

Cytoplasmic RNA was isolated from infected cells, as described above, and 25 μg was hybridized to a single stranded oligonucleotide primer specific for the major late transcription unit. Extensions were performed with AMV reverse transcriptase, and the products analyzed essentially as described previously (Reach *et al.*, 1990). Products extended to the transcription start site should be 36 bases long.

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References

Berk,A.J. (1986) *Annu. Rev. Genet.*, **20**, 45–79.
Berk,A.J. and Schmidt,M.C. (1990) *Genes Dev.*, **4**, 151–155.
Brunet,L.J., Babiss,L.E., Young,C.S.H. and Mills,D.R. (1987) *Mol. Cell. Biol.*, **7**, 1091–1100.
Carcamo,J., Maldonado,E., Cortes,P., Ahn,M.-H., Ilho-Ha, Kasai,Y., Flint,J. and Reinberg,D. (1990) *Genes Dev.*, **4**, 1611–1622.
Chodosh,L.A., Carthew,R.W. and Sharp,P.A. (1986) *Mol. Cell. Biol.*, **6**, 4723–4733.

Chodosh,L.A., Baldwin,A.S., Carthew,R.W. and Sharp,P.A. (1988a) *Cell*, **53**, 11–24.
Chodosh,L.A., Olesen,J., Hahn,S., Baldwin,A.S., Guarente,L. and Sharp,P.A. (1988b) *Cell*, **53**, 25–35.
Concino,M.F., Lee,R.F., Merryweather,J.P. and Weinmann,R. (1984) *Nucleic Acids Res.*, **12**, 7423–7433.
Crossland,L.D. and Raskas,H.J. (1983) *J. Virol.*, **46**, 737–748.
Garfinkel,S., Thompson,J.A., Jacob,W.F., Cohen,R. and Safer,B. (1990) *J. Biol. Chem.*, **265**, 10309–10319.
Giard,D.J., Aaronson,S.A., Todaro,G.J., Arnstein,P., Kersey,J.H., Dosik,H. and Parks,W.P. (1991) *J. Natl Cancer Inst.*, **51**, 1417–1423.
Graham,F.L. and Van der Eb,A.J. (1973) *Virology*, **52**, 456–467.
Gregor,P.D., Sawadogo,M. and Roeder,R.G. (1990) *Genes Dev.*, **4**, 1730–1740.
Hen,R., Sassone-Corsi,P., Corden,J., Gaub,M.P. and Chambon,P. (1982) *Proc. Natl Acad. Sci. USA*, **79**, 7132–7136.
Hu,S.-L. and Manley,J.L. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 820–824.
Jansen-Durr,P., Mondésert,G. and Kédinger,C. (1989) *J. Virol.*, **63**, 5124–5132.
Kambadur,R., Culotta,V. and Hamer,D. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 9168–9172.
Kunkel,T.A. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 488–492.
Lawrence,W.C. and Ginsberg,H.S. (1967) *J. Virol.*, **1**, 851–867.
Lee,R.F., Concino,M.F. and Weinmann,R. (1988) *Virology*, **165**, 52–56.
Lennard,A.C. and Egly,J.M. (1987) *EMBO J.*, **6**, 3027–3034.
Leong,K., Brunet,L. and Berk,A.J. (1988) *Mol. Cell. Biol.*, **8**, 1765–1774.
Leong,K., Lee,W. and Berk,A.J. (1990) *J. Virol.*, **64**, 51–60.
Lewis,E.D. and Manley,J.L. (1985) *Mol. Cell. Biol.*, **5**, 2433–2442.
Logan,J. and Shenk,T. (1986) *Nucleic Acids Res.*, **14**, 6327–6335.
Mansour,J.L., Grodzicker,T. and Tjian,R. (1986) *Mol. Cell. Biol.*, **6**, 2684–2694.
Marsh,J.L., Erfle,M. and Wykes,E.J. (1984) *Gene*, **32**, 481–485.
Meisterernst,M., Horikoshi,M. and Roeder,R.G. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 9153–9157.
Melton,D.A., Krieg,P.A., Rebagliatti,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res*, **12**, 7035–7070.
Mermelstein,F.H., Flores,O. and Reinberg,D. (1989) *Biochim. Biophys. Acta*, **1009**, 1–10.
Moncollin,V., Kempf,A. and Egly,J.-M. (1990) *J. Virol.*, **64**, 3199–3206.
Nakajima,N., Horikoshi,M. and Roeder,R.G. (1988) *Mol. Cell. Biol.*, **8**, 4028–4040.
Natarajan,V., Madden,M.J. and Salzman,N.P. (1985) *J. Virol.*, **55**, 10–15.
Nevins,J.R. (1981) *Cell*, **26**, 213–220.
Philipson,L. (1961) *Virology*, **15**, 263–268.
Pugh,B.F. and Tjian,R. (1990) *Cell*, **61**, 1187–1197.
Reach,M., Babiss,L.E. and Young,C.S.H. (1990) *J. Virol.*, **64**, 5851–5860.
Roovers,D.J., Young,C.S.H., Vos,H.L. and Sussenbach,J.S. (1990) *Virus Genes*, **4**, 53–61.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
Sawadogo,M. and Roeder,R.G. (1985) *Cell*, **44**, 165–175.
Smale,S.T. and Baltimore,D. (1989) *Cell*, **57**, 103–113.
Smale,S.T., Schmidt,M.C., Berk,A.J. and Baltimore,D. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 4509–4513.
Sokal,R.R. and Rohlf,F.J. (1969) *Biometry. The Principles and Practice of Statistics in Biological Research*. W.H.Freeman, San Francisco.
Taylor,I.C.A. and Kingston,R.E. (1990) *Mol. Cell. Biol.*, **10**, 165–175.
Thomas,G.P. and Mathews,M.B. (1980) *Cell*, **22**, 523–533.
Volkert,F.C., Munz,P.L. and Young,C.S.H. (1989) *Virology*, **173**, 77–88.
Wobbe,C.R. and Struhl,K. (1990) *Mol. Cell. Biol.*, **10**, 3859–3867.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–119.
Zagursky,R.J., Baumeister,K., Lomax,N. and Berman,M.L. (1985) *Gene Anal. Tech.*, **2**, 89–94.

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