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Replication-induced stimulation of the major late promoter of adenovirus is correlated to the binding of a factor to sequences in the first intron

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ABSTRACT

The sequence requirements for transcriptional stimulation of the adenovirus major late promoter (MLP) by the products of the early transcription unit E1a and by the replication of viral DNA were analyzed by in vitro transcription. Sequences upstream of +33 are involved in the moderate E1a-responsiveness of the MLP, while sequences between +33 and +131 are required for its major replication-induced transcriptional activation. Dnase I footprinting experiments delineate a sequence component, extending from +76 to +120, which binds protein(s) only in extracts of cells where viral DNA replication occurred. Taken together, these results suggest that the replication-dependent stimulation of the MLP is mediated by the increased binding of this protein(s).

INTRODUCTION

Gene expression is controlled at the level of transcription initiation by a number of mechanisms, all of which require appropriate interactions between specific protein factors and corresponding sequence elements (1-3, and references therein). The regulation of viral genes during lytic infection of animal cells constitutes an attractive model-system for the study of the mechanisms involved in eukaryotic gene control (4).

During lytic infection, adenovirus early genes are first activated by the products of the immediate early gene E1a (5, 6), while other viral genes, such as the protein IX and IVa2 genes and the major late transcription unit, are activated essentially during the late phase of infection, after viral DNA replication has started (7, 8). The molecular process underlying the induction of these intermediate and late genes is still unclear, although the promotor elements required for their transcription have been extensively studied.

Two sequence elements essential for the activity of the major late promotor (MLP) have been identified by site-directed mutagenesis followed by infection (9-11) or transfection (12, 13) analyses and in vitro transcription

experiments (12, 14-17). These elements correspond to the MLP upstream element positioned between -66 and -55, and to the TATA box region, between -31 and -25, relative to the MLP cap site. Distinct protein factors binding to these elements have been identified and extensively purified. These factors correspond to USF (18), MLTF (17) or UEF (19) for the upstream element and to TFIID (20) or BTF 1 (19) for the TATA box region.

Transient expression studies have suggested that MLP activity is stimulated by the products of the viral E1a gene and that the MLP sequences implicated are located between -66 and +33 relative to the MLP cap site (13). In addition, it has been shown that transcription from the MLP is greatly activated by the replication of the viral DNA in infected cells (7). The molecular basis for this replication dependent transcriptional activation is presently unknown. The infection and transfection experiments of Mansour et al. (10), using replicating adenovirus-SV40 hybrid viruses or plasmids, suggested however that a control element, as yet unidentified but located downstream of the MLP cap site, could be implicated in this mechanism.

To get further insight into the mechanism of the transcriptional stimulation of the MLP, we have adapted an *in vitro* transcription system (21) based on extracts from cells which had been infected with wild type Ad 5 or its E1a-deficient d1 312 derivative (6). Comparative transcription analysis of a series of MLP deletion mutants in these extracts indicate that sequences upstream of +33 are required *in vitro* for the transactivation by the E1a proteins, in agreement with the reported *in vivo* sequence dependence (13). Using the DNA replication inhibitor cytosine arabinoside, we also show that most of the *in vitro* transcriptional stimulation of the MLP is replication-dependent, and that the sequences involved are located between +33 and +131. Dnase I footprinting experiments delineate a sequence component, extending from +76 to +120, i.e. within the first intron of the major late transcription unit (22), which binds a protein exclusively active in extracts from cells where viral DNA replication occurred.

MATERIALS AND METHODS

Preparation of whole cell extracts

HeLa cells were infected with wt Ad5 or d1 312 at 10 pfu/cell. Cells were grown in Eagle's medium supplemented with 5% calf serum. For replication-blocked extracts infected cells were grown in the presence of cytosine arabinoside (AraC) at a final concentration of 20 µg/ml, which was maintained by adding fresh AraC after 12 hours (21). Extracts were prepared from these

cells according to Manley et al. (23) except that final dialysis was against a buffer (buffer A; ref. 24) containing 50 mM Tris-HCl pH 7.9, 12.5 mM $MgCl_2$, 40 mM $(NH_4)_2SO_4$, 0.1 mM EDTA, 2 mM DTT, 17% glycerol. If necessary, protein concentration of the extracts was adjusted to 6 µg/µl after dialysis.

In vitro transcription assays.

Transcription was carried out in 24 µl final volume using 12 µl of extract, 100 to 400 ng of template DNA digested with appropriate restriction endonucleases, unlabelled ATP, GTP and UTP at 250 µM each, unlabelled CTP at 12.5 µM and 0.3 µCi of α-[^{32}P]-labelled CTP (specific activity 600 Ci/mmol). The template was preincubated with the extract for 10 min at 25°C before adding the nucleotides. Reactions were carried out for 45 min at 30°C. After synthesis, RNA was extracted and analysed by 5% polyacrylamide-urea gel electrophoresis. Quantitation of specific transcripts was achieved by scanning of the autoradiograms (standard deviations calculated from independent experiments were in the range of 10 to 20%).

Labeling of DNA fragments.

pML553 DNA was cut by HindIII and subsequently either 3'-end labeled by the Klenow polymerase using α-[^{32}P]-dATP (3000 Ci/mmol) or 5'-end labeled by T4 polynucleotide kinase using γ-[^{32}P]-ATP (3000 Ci/mmol). The labeled DNA was cut by BamHI, the 454 bp BamHI/HindIII fragment was isolated by preparative gel electrophoresis and electroeluted.

Footprinting reactions.

Footprinting reactions were done essentially as described (25) : 0.5-1 ng the labelled fragment in the presence of 100 ng of poly(dI·dC) was incubated with different amounts of extract protein for 10 min at 30°C. Incubation was in the same buffer as *in vitro* transcription, i.e. buffer A, diluted 1:1. DNAse I (Sigma, 1550 Kunitz units/mg protein) was added, the incubation continued for 5 min at 30°C. The DNA was purified, heat-denatured and loaded on a 8% polyacrylamide gel containing 50% urea. Gels were dried and exposed to Kodak XR film for autoradiography.

RESULTS

Sequences upstream as well as downstream of the cap site contribute to the transcriptional stimulation of the MLP

In an earlier *in vitro* study, Leong and Berk (21) have shown that exogenous MLP sequences are transcribed more efficiently in extracts prepared from cells infected with wild type adenovirus-5 (wt) than from mock-infected cells or cells infected with the E1a-deletion mutant dl 312 (dl). In addition

these authors observed that the differential activity of these extracts was most pronounced when prepared at 20 hours post-infection (hpi). To identify the MLP sequences involved in this transcriptional stimulation we have used 20 hpi extracts to compare the template efficiencies of a series of MLP restriction fragments, in run-off transcription experiments.

Whole cell extracts were prepared 20 hpi according to Manley et al. (23) from HeLa cells infected at 10 pfu/cell with wt or dl 312 virus and are referred to hereafter as the 20 hpi wt or dl extracts, respectively. The extracts were first assayed for in vitro transcriptional activity using as templates the AvaII-cut plasmid pML553 (run-off transcript 451 nt, cf. Fig. 1A) and the BamHI-linearized plasmid pG containing the rabbit beta-globin gene (26; run-off transcript 475 nt). A typical result presented in Fig. 2A, shows that the MLP is transcribed about 20-30-fold more efficiently in 20 hpi wt extracts than in 20 hpi dl extracts. The stimulation is most

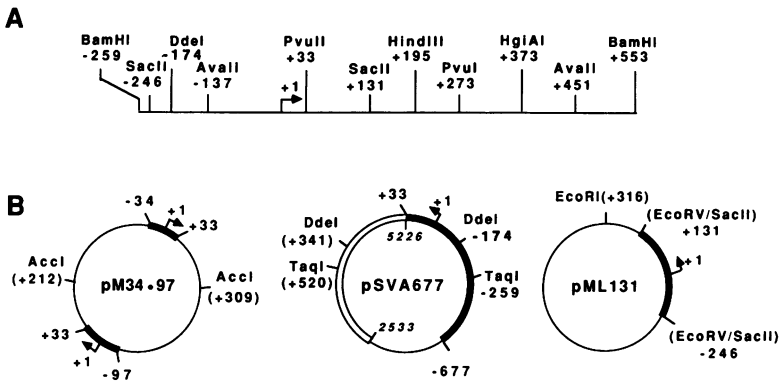

Figure 1. Structure of the MLP-containing recombinants used as templates

A. Restriction map of the MLP fragment contained in pML553. The TaqI fragment of Ad2 DNA (-259 to + 553) containing the MLP cap site was repaired and inserted into BamHI-cleaved repaired pBR322 vector DNA. Cleavage sites in the MLP insert of pML553 for selected restriction endonucleases are indicated and positioned with respect to the MLP start site (+1). The arrow points to the direction of transcription.

B. Schematic drawing of pM34.97 (ref. 16), pSVA677 (formerly called pSVA500 in ref. 12), and pML131. pML131 was constructed by inserting the SacII fragment containing the MLP cap site in EcoRV-cleaved pBR322 vector DNA. Closed boxes indicate MLP sequences, the open box in pSVA677 indicates SV40 early coding sequences (between coordinates 5226 and 2533), thin lines represent vector sequence. Coordinates of relevant restriction sites are given relative to the MLP start site (+1). Restriction sites in parentheses were lost during cloning. The coordinates of sites used for run-off transcription are given in parentheses.

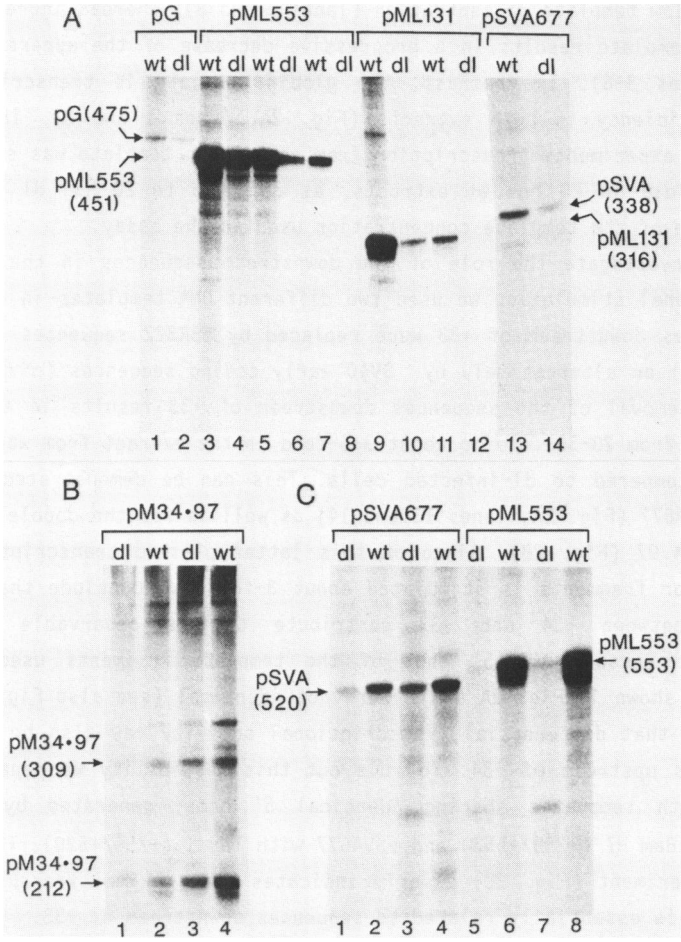

Figure 2. Run-off transcription analysis of MLP activity in extracts prepared at 20 hpi

A. Transcription was carried out in the presence of 20 hpi wt extracts (wt) or 20 hpi dl extracts (dl), with 100 ng of BamHI- cut pG (26) containing the rabbit beta-globin gene (lanes 1, 2), 100 ng (lanes 7, 8), 200 ng (lanes 5, 6), or 400 ng (lanes 3, 4) of AvaII-cut pML553, 100 ng (lanes 11, 12) or 200 ng (lanes 9, 10) of pML131, cut by AvaII and EcoRI, 100 ng (lanes 13, 14) of pSVA677, cut by DdeI. For lanes 13 and 14 a 3-fold longer exposure of the autoradiogram is shown. The arrows point to specific RNA transcribed from the indicated template with the expected run-off size given in parentheses.

B. Transcription was carried out as in panel A, but with 100 ng (lanes 1, 2) or 200 ng (lanes 3, 4) of the AccI-cut double promoter plasmid pM34.97.

C. Transcription was carried out as in panel A, but with 100 ng (lanes 1, 2, 5, 6) or 200 ng (lanes 3, 4, 7, 8) of the TaqI-cut pSVA677 (lanes 1-4) or the BamHI-cut pML553 (lanes 5-8) plasmids.

evident at low template concentration (lanes 7 and 8), whereas increasing the amount of template results in a progressive decrease of the apparent stimulation (lanes 3-6). In contrast, the globin template is transcribed with similar efficiency in both extracts (Fig. 2A, lanes 1 and 2). In several independent experiments transcription from the globin template was stimulated at most 3-fold in 20 hpi wt extracts, as compared to 20 hpi dl extracts, irrespective of the template concentration used in the assay.

To investigate the role of the downstream sequences in the observed transcriptional stimulation we used two different DNA templates in which the MLP sequences downstream of +33 were replaced by pBR322 sequences (pM34.97, see Fig. 1B) or alternatively by SV40 early coding sequences (pSVA677, see Fig. 1B). Removal of the sequences downstream of +33 results in a drop of stimulation from 20-30-fold to about 3-5-fold in the extract from wt infected cells, as compared to dl-infected cells. This can be demonstrated for the plasmid pSVA677 (Fig. 2A, lanes 13 and 14) as well as for the double promotor plasmid pM34.97 (Fig. 2B). Since on this latter plasmid transcription from both promotor fragments is stimulated about 3-fold, we conclude that mainly sequences between -34 and +33 contribute to the observable residual stimulation. Because the 5' ends of the template fragments used in the experiments shown in Fig. 2A and B were not identical (see also Fig. 4), one could argue that differential transcriptional activity may also be affected by sequences upstream of -34. To rule out this possibility we repeated the analysis with templates sharing identical 5' ends, generated by cutting pML553 with Bam HI (-259/+553) and pSVA677 with Taq I (-259/+520). The result of this experiment (Fig. 2C) clearly indicates that maximal transcriptional stimulation is essentially related to sequences downstream of +33.

To further define the downstream sequences required for the observed stimulation of MLP transcription we deleted various amounts of these sequences by cutting the plasmid pML553 with AvaII (at positions -137 and +451 relative to the MLP cap site) and various other restriction endonucleases recognizing sites in the downstream portion of the MLP (cf. Fig. 1A). By analyzing these templates in transcription assays we found that the stimulation of MLP-specific transcription from templates cut with AvaII and with HgiAI (+373), PvuI (+273) or HindIII (+195), respectively, was indistinguishable from that observed with the template cut with AvaII alone (data not shown but summarized in Fig. 4). To avoid the difficulty of detecting short run-off transcripts, for further deletion analysis we subcloned fragments of the promotor. First we cloned the HindIII fragment of pML553 (extending

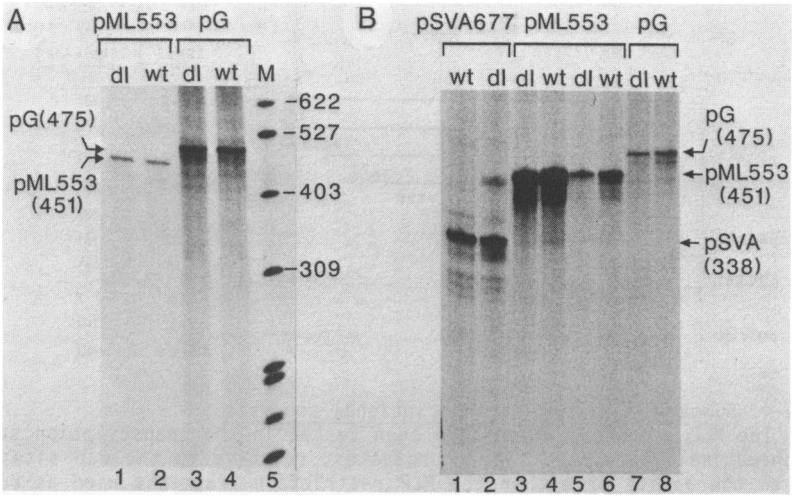

Figure 3. Transcriptional activity of extracts prepared from infected cells in which viral DNA replication was not occuring

A. 6 hpi wt (wt, lanes 2, 4) and dl extracts (dl, lanes 1, 3) were tested using 100 ng of AvaII-cut pML553 (lanes 1, 2) or 100 ng of the BamHI-cut pG plasmid (lanes 3, 4). Lane 5 : MspI-digested pBR322 DNA.

B. Replication-blocked 20 hpi wt (wt, lanes 1, 4, 6, 8) and dl extracts (dl, lanes 2, 3, 5, 7) were tested using 200 ng of DdeI-cut pSVA677 (lanes 1, 2), 100 ng (lanes 5, 6) or 200 ng (lanes 3, 4) of AvaII-cut pML553 and 100 ng of BamHI-cut pG (lanes 7, 8).

between positions -605 and +195 with respect to the MLP cap site) into the HindIII site of pBR322. This template, cut with AvaII (position -137) and EcoRV (position +310 relative to the MLP cap site) was about 20-30-fold more efficiently transcribed in 20 hpi wt extracts compared to 20 hpi dl extracts (see Fig. 4). Finally we cloned the SacII fragment of the MLP (extending between -246 and +131, see Fig. 1A) in the EcoRV site of pBR322. Transcription from the resulting plasmid pML131 (Fig. 1B), cut with AvaII (position -137) and EcoRI (position +316 relative to the MLP cap site), again was about 20-30 times more efficient in the 20 hpi wt extract (Fig. 2A, lanes 9-12). Taken together (see Fig. 4) these results indicate that the downstream sequences required for maximal transcriptional stimulation from the MLP are located between +33 and +131.

The stimulatory effect of MLP downstream sequences is dependent on viral DNA replication before extract preparation

Since at 20 hpi the wt virus has replicated, whereas the E1a-deficient mutant has not (6, 27), the observed stimulation could be caused either by

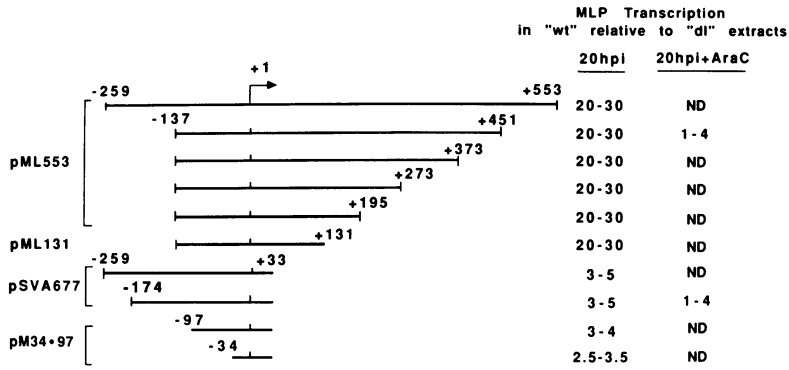

Figure 4. Summary of the MLP transcriptional analysis.
The MLP sequences which have been tested in the transcription studies are schematically depicted. The coordinates, relative to the cap site (+1), refer to the limits of either the MLP restriction fragments used as run-off templates (barred ends) or the MLP segments present within larger run-off template fragments (free ends). The names of the corresponding plasmids are given on the left. The tabulated values indicate for each template the ratio of the MLP-specific transcription in 20 hpi wt extracts to that in 20 hpi dl extracts, prepared from non-treated (20 hpi) or replication-blocked (20 hpi + AraC) cells. The ranges of values correspond to the upper and lower limits of these ratios as derived from series of 3-5 independent experiments similar to, and including those shown in Fig. 2 and 3. Only the results of transcriptions performed in the presence of 100 ng of template are presented. ND, not determined.

the selective expression of the E1a gene in wt-infected cells or by the replication of the viral DNA in these cells. To distinguish between these alternatives we first prepared extracts from dl and wt-infected cells, harvested at 6 hpi, a time at which neither the dl nor the majority of the wt viral DNA molecules have undergone replication. When these extracts were tested for their transcriptional activity (Fig. 3A, lanes 1-4), the difference between dl and wt 6 hpi extracts never exceeded 3-fold, regardless of the template (MLP or globin) used.

To further examine the effect of viral DNA replication prior to extract preparation, we grew wt-infected or dl-infected cells in the presence of cytosine arabinoside (AraC) to prevent DNA replication (21). When we probed for MLP expression in these cells we found a dramatic decrease of detectable MLP transcripts in the cytoplasmic RNA of these cells, as compared to wt-infected cells grown in the absence of AraC (data not shown). This indicates that viral DNA replication has been blocked efficiently. Extracts were prepared from these cells at 20 hpi (called hereafter replication-blocked wt or dl extracts) and assayed for transcription. Transcription

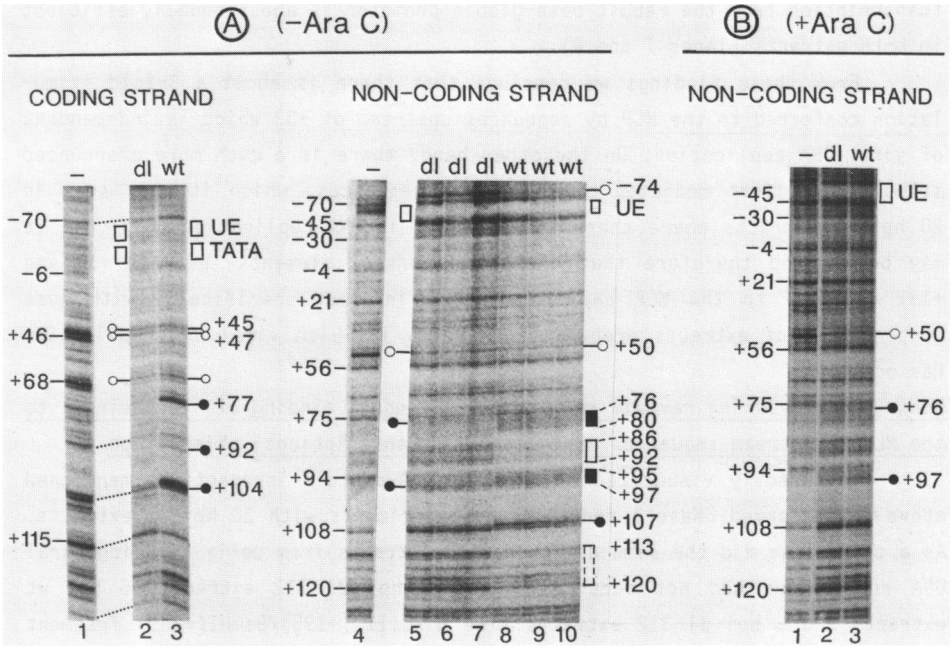

Figure 5. Comparative Dnase I footprinting on the MLP downstream region.

A. Extracts prepared from non-treated (-AraC) cells : 60 µg of either 20 hpi wt extract (wt, lanes 3, 8, 9, 10) or 20 hpi dl extract (dl, lanes 2, 5, 6, 7) were incubated with 1 ng of the BamHI/HindIII fragment of the MLP (see Fig. 1A) either 3'end-labelled (non-coding strand) or 5'end-labelled (coding strand, i.e. transcribed strand) at the HindIII site. Digestion was with 750 ng (lanes 2, 3), 900 ng (lane 8), 1000 ng (lanes 7, 9), 1200 ng (lanes 6, 10) or 1400 ng (lane 5) of Dnase I. (-) Naked DNA. Symbols next to the dl and wt lanes highlight the effects of the corresponding extracts on the Dnase I digestion pattern: filled symbols, hypersensitive sites; open symbols, protected sites. Nucleotide positions are relative to the MLP capsite. UE and TATA stand for MLP upstream element and TATA box, respectively. The digestion pattern of lane 1, which was run next to lane 3 on the gel, is correlated to the patterns of lanes 2 and 3 by the dotted lines.

B. Extracts prepared from replication-blocked (+AraC) cells : 60 µg of either replication-blocked wt extract (wt, lane 3) or replication-blocked dl 312 extract (dl, lane 2) were incubated with 1 ng of the same fragment as in panel A and digested with 850 ng (lanes 2, 3) of Dnase I.

from the complete MLP, i.e. pML553 cut by AvaII, was stimulated about 3-fold in replication-blocked wt extracts compared to replication-blocked dl extracts (Fig. 3B, lanes 3-6). About the same degree of stimulation is observed for the template pSVA677, cut by DdeI (lanes 1 and 2), while

transcription from the rabbit beta-globin promotor is about equally efficient in both extracts (lanes 7 and 8).

From these findings we conclude that there is about a 3-fold stimulation conferred to the MLP by sequences upstream of +33 which is independent of viral DNA replication. On the other hand, there is a much more pronounced stimulatory effect mediated by downstream sequences, which is only seen in 20 hpi wt extracts where there was no block in DNA replication (Fig. 4). It may be deduced therefore that there are control elements, between +33 and +131 relative to the MLP cap site, which interact specifically with some component(s) of extracts prepared from cells in which viral DNA replication has occurred.

Dnase I footprinting reveals replication-dependent binding of a protein(s) to the MLP downstream sequences implicated in transcriptional stimulation

To directly visualize the putative DNA-protein interactions mentioned above we performed DNase I footprinting experiments with 20 hpi wt extracts. As a control we did the same analysis with extracts from cells in which viral DNA replication had not occurred, i.e. 20 hpi dl 312 extracts, 6 hpi wt extracts and 6 hpi dl 312 extracts. The HindIII (+195)/BamHI(-259) fragment of pML553 (see Fig. 1A) encompassing the MLP cap site, was either 3'-end labeled (non-coding strand) or 5'-end labeled (coding strand) at the HindIII site. The labeled fragment was incubated with equal amounts of protein from different extracts and digested by DNase I.

Comparing the protection pattern of extracts prepared at 20 hpi from either wt or dl-infected cells, the following features are found in common to both extracts (Fig. 5A) : a significant protection of regions corresponding to the MLP upstream element and a weaker one on the TATA box, respectively (18); minor protections around position +50 on the non-coding strand and positions +45, +47 and +68 on the coding strand; a hypersensitive site at +97 on the non-coding strand.

With the 20 hpi wt extract one additional protection is observed, which is not detected with the 20 hpi dl extract. The corresponding protein binding site is characterized by the occurence of several hypersensitive sites (+77, +92, +104) on the coding strand and a protected region between +86 and +92 on the non-coding strand, flanked by hypersensitive regions. A weak protection, between +113 and +120 on the non-coding strand, is also detected, selectively with 20 hpi wt extracts. Furthermore we detect a slight but reproducible difference in the footprint on the upstream element, where position -74 is protected selectively in 20 hpi wt extracts. In an attempt to

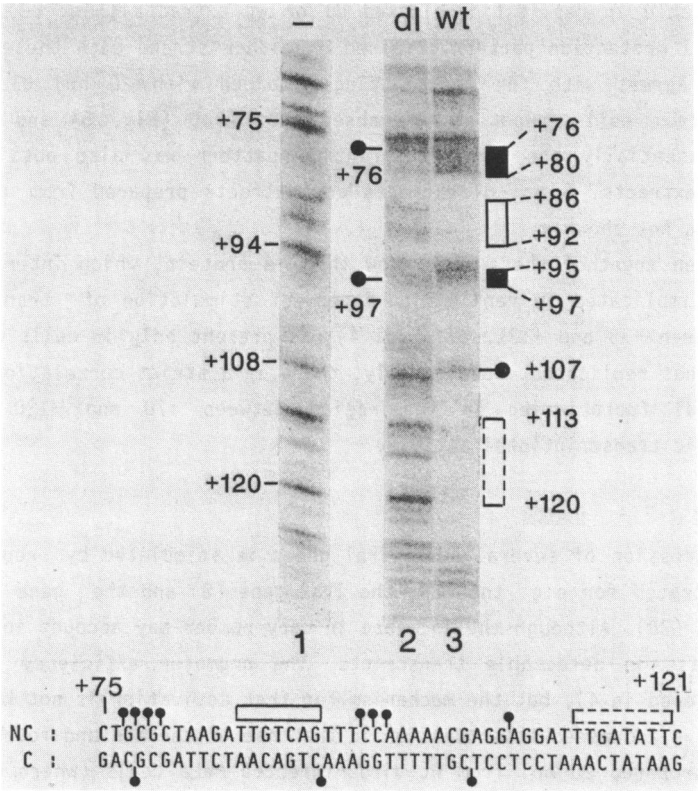

Figure 6. Detailed analysis of the replication-induced Dnase I footprint on the MLP downstream region.
60 µg of either 20 hpi dl (dl, lane 2) or 20 hpi wt extract (wt, lane 3) were incubated with 0.5 ng of the MLP probe fragment labelled at the non-coding strand (see Fig. 5), and digested with 600 ng (lane 3) and 700 ng (lane 2) of Dnase I. Symbols alongside the auto-radiograms are as in Fig. 5. The nucleotide sequence of the relevant MLP region is shown, with the symbols indicating protected or hyper-sensitive nucleotides on both the non-coding (NC) and coding (d) strands.

better visualize the differential protection, we performed footprinting reactions at a higher protein/DNA ratio. Under these conditions (Fig. 6) protections of both regions +86/+92 and +113/+120 are clearly visible reflecting the excess of binding protein used in this experiment.

To elucidate the effect of DNA replication on the DNA-protein interactions we analyzed the footprints obtained with replication blocked dl extracts and replication-blocked wt extracts. Clearly the protection of region +86/+92 on the non-coding strand, as seen in 20 hpi wt extracts, is

not detectable in replication-blocked dl or wt extracts (Fig. 5B). In fact the Dnase I protection pattern obtained on either strand with these extracts perfectly agrees with the footprinting obtained with 20 hpi dl extracts prepared from cells grown in the absence of AraC (Fig. 5A and data not shown). Essentially the same footprinting pattern was also obtained with 6 hpi wt extracts, 6 hpi dl extracts and extracts prepared from uninfected cells (data not shown).

Taken together these data show that a protein, which interacts with sequences implicated in replication-dependent stimulation of transcription (i.e. between +33 and +131, cf. Fig. 4), is present only in cells where the viral DNA has replicated. Specifically, there is a strict correlation between differential footprinting in the region between +76 and +120 and the MLP-specific transcriptional activity.

DISCUSSION

Expression of several adenoviral genes is stimulated by replication, as demonstrated for e.g. the MLP, the IVa2 gene (8) and the gene for viral protein IX (28). Although the increase in copy number may account in part for the increase in detectable transcripts, the promoter efficiency increases also (reviewed in 4), but the mechanism for that activation is not known.

We have addressed this question in the case of the MLP and found that in extracts prepared 20 hpi from wt virus-infected HeLa cells (where viral DNA has replicated) transcription from the MLP is about 20-30-fold more efficient than in extracts from dl 312 (dl)-infected cells (where viral DNA has not replicated). By contrast, when viral DNA replication was blocked, the difference between 20 hpi wt and 20 hpi dl extracts for MLP-specific transcription was considerably reduced. Deletional analysis indicated that the replication-dependent stimulation of MLP transcription is mediated by sequences between +33 and +131. A residual stimulation of about 3-fold occurs independent of these downstream sequences, but involving sequences comprised between -34 and +33. Since this effect is seen also in extracts from cells where viral replication was blocked, it may be mediated by the viral E1a gene products selectively expressed in wt-infected cells, as has been concluded by Leong and Berk (21) using a similar *in vitro* transcription system. In contrast to these authors, however, we found that the extracts from wt-infected cells show only very poor transcriptional stimulation of the MLP compared to extracts from dl-infected cells, if the cells were grown in the presence of AraC. Similarly, Leff and Chambon (29) failed to detect a pronounced stimulation of MLP

transcription by E1a in transient expression experiments. The low effect of the E1a products on MLP activity may actually correspond to the basal level of E1a-responsiveness shared by some cellular promoters, like the globin or conalbumin gene promoters (26, 30, 31) and most likely only involving their TATA box and cap site regions (30, 32).

In vivo MLP expression is stimulated about thousandfold by viral DNA replication (7). Results of double infection experiments (33) demonstrate that this effect requires replication of the viral template and cannot be ascribed solely to a readily diffusible trans-acting factor. This conclusion is supported by the results of Mansour et al. (10), which indicate that, in a transfection assay, transcription from MLP-containing templates was stimulated only on plasmids containing an origin of replication (SV40 ori region) and only in cells supporting replication, i.e. Cos-7 cells. However replication of the template is not by itself sufficient for the transcriptional stimulation, since it was shown by these authors that this stimulation occurs only in adenovirus-infected Cos-7 cells, suggesting that virus-induced factors are also required. In the same study the authors show that this in vivo replication dependent stimulation of the MLP is mediated by an element the 3' border of which is located between +33 and +195. In this respect the results of our in vitro experiments are in agreement with the in vivo situation. On the other hand, replication of the template itself does not seem to be required in the in vitro system, since immediate transcriptional stimulation occurs on exogenous templates after a short preincubation period. We assume that the transcriptionally active complex is readily formed under our in vitro conditions, whereas its formation might be prevented in vivo, due to some, as yet undefined, restriction which would be abolished by template replication. Furthermore, it is not excluded that only part of the in vivo MLP induction is revealed in the present in vitro experiments and that coupled in vitro replication-transcription systems will display still higher MLP activities.

From the in vitro transcription experiments it appears that downstream sequences are implicated in replication-dependent stimulation of the MLP. Applying Dnase I footprinting we examined whether positive or negative regulation may account for the observed difference in the level of MLP- specific transcription. We never detected any difference in the abundance of the factors required for constitutive transcription (UEF and TATA box binding factors) in different dl extracts vs. wt extracts (data not shown, cf. Fig. 4). Interestingly, we found binding of a protein to sequences between

+86 and +92 as well as a weaker footprint between +113 and +120, both of which are detected exclusively in extracts prepared from wt-infected cells after viral DNA replication. These protections represent the only differential footprints in the region essential for replication-dependent transcriptional stimulation of the MLP, i.e. between +33 and +131, suggesting therefore that the observed stimulation is caused by induction and/or activation of a positive transcription factor.

Recently partial purification from noninfected cells of a transcription factor acting through downstream sequences has been described (34). Whereas transcription from a MLP template extending to +536 depends on this factor, there is no requirement for this factor to transcribe templates lacking MLP sequences downstream of +11. The footprint we observed in the region +86/+92 with 20 hpi wt extracts most probably is not related to the factor identified by Reinberg et al. (34), since it is not detectable in extracts from uninfected cells (our unpublished data).

From our present results, it is tempting to speculate that the replication-dependent activation of MLP transcription is due to the binding of a particular cellular or viral factor required for both replication and late gene expression. The host-coded nuclear factor I (NFI) is a representative example of such bifunctional proteins, since it has been implicated in adenovirus DNA replication as well as in transcription of different cellular and viral genes (35). Because no footprint on the MLP downstream element is detected in uninfected HeLa cells, it is likely, however, that the activity of the cognate binding factor (NFI or others) is induced in the course of viral DNA replication.

Besides the adenovirus major late gene, a number of other viral (36-39) or cellular (40-47) genes have been found to contain regulatory elements, located downstream of the transcription start site. It is striking that in these cases the downstream elements are essential for efficient cell-specific expression.

A precise mutational analysis, presently in progress, will help refining the delineation of the MLP replication-dependent promoter element. Further characterization of the relevant factor(s) will be gained by following its induction during viral infection and ultimately by its purification from infected cells.

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REFERENCES

1. Dynan, W. and Tjian, R. (1985). Nature 316, 774-778.
2. Serfling, E., Jasin, M. and Schaffner W. (1985). Trends in Genet. 1, 224-230.
3. Ptashne, M. (1986). Nature 322, 697-701.
4. Berk, A.J. (1986). Ann. Rev. Genet. 20, 45-79.
5. Berk, A.J., Lee, F., Harrison, T., Williams, J. and Sharp, P.A. (1979). Cell 17, 935-944.
6. Jones, N. and Shenk, T. (1979). Proc. Natl. Acad. Sci. USA 76, 3665-3669.
7. Shaw, A.R. and Ziff, E.B. (1980). Cell 22, 905-916.
8. Crossland, L.D. and Raskas, H.J. (1983). J. Virol. 46, 737-748.
9. Logan, J. and Shenk. T. (1986). Nucleic Acids Res. 14, 6327-6335.
10. Mansour, S.L., Grodzicker, T. and Tjian, R. (1986). Mol. Cell. Biol. 6, 2684-2694.
11. Brunet, L.J., Babiss, L.E., Young, C.S.H. and Mills, D.R. (1987). Mol. Cell. Biol. 7, 1091-1100.
12. Hen, R., Sassone-Corsi, P., Corden, J., Gaub, M-P. and Chambon, P. (1982). Proc. Natl. Acad. Sci. USA 79, 7132-7136.
13. Lewis, E.D. and Manley, J.L. (1985). Mol. Cell. Biol. 5, 2433-2442.
14. Hu, S.-L. and Manley, J.L. (1981). Proc. Natl. Acad. Sci. USA 78, 820-824.
15. Concino, M.F., Lee, R.F., Merryweather, J.P. and Weinmann, R. (1984). Nucleic Acids Res. 12, 7425-7433.
16. Miyamoto, N.G., Moncollin, V., Egly, J.M. and Chambon, P. (1985). EMBO J. 4, 3563-3570.
17. Chodosh, L.A., Carthew, R.W. and Sharp, P.A. (1986). Mol. Cell. Biol. 6, 4723-4733.
18. Sawadogo, M. and Roeder, R.G. (1985). Cell 43, 165-175.
19. Moncollin, V., Miyamoto, N.G., Zheng, X.M. and Egly, J.M. (1986). EMBO J. 5, 2577-2584.
20. Sawadago, M. and Roeder, R.G. (1985). Proc. Natl. Acad. Sci. USA 82, 4394-4398.
21. Leong, K. and Berk, A.J. (1986). Proc. Natl. Acad. Sci. USA 3, 5844-5848.
22. Zain, S., Sambrook, J., Roberts, R.J., Keller, W., Fried, M. and Dunn, A.R. (1979). Cell 16, 851-861.
23. Manley, J.L., Fire, A., Samuels, M. and Sharp, P.A. (1983). Methods in Enzymology 101, 568-592.
24. Natarajan, V., Madden, M.J. and Salzman, N.P. (1984) Proc. Natl. Acad. Sci. USA 81, 6290-6294.
25. Boeuf, H., Zajchowski, D.A., Tamura, T., Hauss, C. and Kédinger, C. (1987). Nucleic Acids Res. 15, 509-527.
26. Jalinot, P. and Kédinger, C. (1986). Nucleic Acids Res. 14, 2551-2569.
27. Devaux, B., Albrecht, G. and Kédinger C. (1987). Mol. Cell. Biol. 7, 4560-4563.

28. Venkatesh, L.K. and Chinnadurai, G. (1987). Nucleic Acids Res. 15, 2235-2250.
29. Leff, T. and Chambon, P. (1986). Mol. Cell. Biol. 6, 201-208.
30. Green, M., Treisman, R. and Maniatis, T. (1983). Cell, 35, 137-148.
31. Zajchowski, D.A., Boeuf, H. and Kédinger, C. (1987). Gene 58, 243-256.
32. Wu, L., Rosser, D.S.E., Schmidt, M.C. and Berk, A. (1987). Nature 326, 512-515.
33. Thomas, G.P. and Mathews, M.B. (1980). Cell 22, 523-533.
34. Reinberg, D., Horikoshi, M. and Roeder, R.G. (1987) J. Biol. Chem. 262, 3322-3330.
35. Jones, K.A., Kadonaga, J.T., Rosenfeld, P.J., Kelly, T.J. and Tjian, R. (1987). Cell 48, 79-89.
36. Derse, D., Caradonna, S.J. and Casey, J.V. (1985). Science 227, 317-320.
37. Rosen, C.A., Sodroski, J.G. and Haseltine, W.A. (1985). Cell 41, 813-823.
38. Muesing, M.A., Smith, D.H. and Capon, D.J. (1987). Cell 48, 691-701.
39. Ohtami, K., Nakamura, M., Saito, S., Noda, T., Ito, Y., Sugamura, K. and Hinuma, Y. (1987). EMBO J. 6, 389-395.
40. Charnay, P., Treisman, R., Mellon, P., Chao, M., Axel, R. and Maniatis, T. (1984). Cell 38, 251-263.
41. Antoniou, M., de Boer, E., Habets, G. and Grosveld, F. (1988). EMBO J. 7, 377-384.
42. Merril, G.F., Hauschka, S.D. and McKnight, S.L. (1984). Mol. Cell. Biol. 4, 1777-1784.
43. Banerji, J., Olson, L. and Schaffner, W. (1983). Cell 33, 729-740.
44. Queen, C. and Baltimore, D. (1983). Cell 33, 741-748.
45. Paek, I. and Axel, R. (1987). Mol. Cell. Biol. 7, 1496-1507.
46. Theill, L.E., Wiborg, O. and Vuust, J. (1987). Mol. Cell. Biol. 7, 4329-4336.
47. Rossouw, C.M.S., Vergeer, W.P., du Ploy, S.J., Bernard, M.P., Ramirez, F. and de Wet, W.J. (1987). J. Biol. Chem. 262, 15151-15157.