The Mammalian Upstream Element Factor Recognizes Two Sites in the Adenovirus Type 2 IVa2-Major Late Promoter Intergenic Region and Stimulates Both Promoters

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The adenovirus type 2 major late upstream element factor (UEF) recognizes two similar elements that lie between the major late promoter (MLP) and IVa2 promoter cap sites (the previously characterized MLP-UE from nucleotides –49 to –67 and the IVa2-UE from nucleotides –98 to –122). DNase I footprinting and gel retention assays showed that the UEF has a lower affinity for the IVa2-UE than for the MLP-UE. In vitro transcription experiments demonstrated first that the IVa2 promoter, which lacks a consensus TATA box, may work, as does the MLP, in the absence of its proximal upstream element and second that the IVa2-UE stimulated IVa2 transcription two- to threefold, as MLP-UE did for the MLP. In addition, we demonstrated that the more distal upstream element has a weak stimulatory effect on transcription of both promoters.

Transcription of protein-coding genes requires the presence of one or more distinct promoter elements in the DNA template. The promoters often have a TATA-homologous sequence located around 30 base pairs upstream from the initiation site. A transcription factor that specifically binds to this sequence has been identified in yeasts (4, 7), drosophila (34), and humans (11, 37). In addition to the TATA box-recognizing factor, BTF1 (also known as TFIID and DB [23, 35]), at least three other factors (STF, BTF2, BTF3) and RNA polymerase B (II) are required for accurate initiation of transcription in vitro (26). Besides these general transcription factors which catalyze the basal level of transcription of various genes, there are some sequence-specific factors which regulate transcription of their corresponding target genes (for a review, see reference 40 and references therein).

The adenovirus type 2 (Ad2) major late promoter (MLP) contains, in addition to the TATA box, an upstream element (MLP-UE) located between –49 and –67 (25). This UE contains a dyad symmetry sequence, CAC/GTG, and is the target of a sequence-specific binding factor called the upstream element factor (UEF) (24), MLTF (5), or USF (37) which stimulates transcription. This UEF was originally identified in HeLa cells, but an analog has been detected in yeasts (1, 8; unpublished data). A UE-like sequence has been found at various locations in several genes of higher eucaryotes including the mouse thymidylate synthase (12) and metallothionein I (6) genes, the rat γ fibrinogen promoter (9), the long terminal repeat of human immunodeficiency virus type 1 (29), and the *Xenopus laevis* histone H2A1 gene (27). This UE-like sequence has also been found in the promoter regions of lower eucaryotic genes such as the *GAL2* (3) and arginine tRNA (39) genes as well as in the centromeric DNA element (2) from yeasts.

The Ad2 IVa2 gene is located 210 base pairs upstream from the ML initiation site and is transcribed from the opposite strand of the viral DNA. Deletion analysis has shown that the IVa2 promoter and the MLP share regulatory sequences (30, 31).

In the present study, we showed by gel retardation and DNase I footprinting experiments that the highly purified UEF is capable of recognizing, in addition to the MLP-UE binding site (from nucleotides –49 to –67), another homologous sequence (IVa2-UE) which lies further upstream (between nucleotides –98 and –122 from the MLP start site). We then demonstrated that the UEF has a lower affinity for the IVa2-UE than for the MLP-UE and that in vitro IVa2 transcription is stimulated by the IVa2-UE, whereas the MLP is stimulated by the MLP-UE.

MATERIALS AND METHODS

Purification of UEF. The UEF was purified from a HeLa whole-cell extract (WCE) (22), and the first three steps of the purification procedure (heparin-Ultrogel, DEAE-5PW, SP-5PW) were as previously described (26). The SP0.35 fraction was dialyzed in buffer A (50 mM Tris hydrochloride [pH 7.9], 50 mM KCl, 8.7% glycerol, 0.1 mM EDTA, 0.5 mM dithiothreitol [DTT]) and incubated for 15 min with poly(dI-dC)-poly(dI-dC) (Pharmacia, Uppsala, Sweden) (100 μ g/ml of fraction) and 4 mM $MgCl_2$ at 4°C before being loaded onto a 1-ml DNA sequence-specific column made as follows. The two 35-mer synthetic oligonucleotide strands (sequence –41 to –71 with respect to the MLP start site with a GATC tetramer added at the 5' extremity) were hybridized and ligated with T4 DNA ligase. The polymers were fixed onto a Sepharose CL-4B resin (Pharmacia) preactivated by CNBr (18). The column was then successively washed with 10 column volumes of buffer B (50 mM Tris hydrochloride [pH 7.9], 50 mM KCl, 17.4% glycerol, 1 mM DTT, 5 mM $MgCl_2$, 0.1% Nonidet P-40) and 5 column volumes of buffer B containing 0.3 M KCl. The UEF was then eluted by 10 column volumes of buffer B containing 1 M KCl. The 1 M KCl fraction (DNA-1M), which contained two major polypeptides of 45 and 42 kilodaltons (kDa), was dialyzed against buffer C (10 mM potassium phosphate [pH 7], 0.01 mM $CaCl_2$, 8.7% glycerol, 0.5 mM DTT) and loaded onto a hydroxylapatite column (500 μ l) (BDH, Poole, England) equilibrated in buffer C. The UEF was eluted by 0.12 M potassium phosphate in buffer C (3 column volumes). The

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fractions were then dialyzed against buffer D (50 mM Tris hydrochloride [pH 7.9], 25% glycerol, 0.5 mM DTT, 0.1 mM EDTA, 50 mM KCl) and stored at -80°C . Upon elution from a sodium dodecyl sulfate-polyacrylamide gel and renaturation (15), both the 45- and 42-kDa polypeptides were able to footprint the MLP-UE (data not shown), thus suggesting that the 42-kDa polypeptide is a proteolytic product derived from the 45-kDa protein. Using the sequence-specific DNA column, the previously described procedure for purifying the UEF was improved up to a 30,000-fold purification factor with a 10% recovery yield (see reference 26 for purification).

In vitro transcription. The in vitro transcription runoff assay consisted of a 15-min preincubation at 24°C in a 20- μl reaction volume containing 25 mM Tris hydrochloride, 2.5 mM MgCl_2 , 20 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1 mM EDTA, 7.5% glycerol, 0.5 mM DTT, 40 ng of poly(dI-dC)-poly(dI-dC), 10 μl of a HeLa WCE, and 50 ng of the indicated truncated DNA template. After the preincubation period, 4 μl of a mixture containing 10 mM $(\text{NH}_4)_2\text{SO}_4$, 24 mM MgCl_2 , 1.25 mM each ATP, GTP, and UTP, and 6.25 μM CTP containing 2 to 5 μCi of $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ was added to start RNA synthesis. The competition assay was as described above except that the indicated competitor DNA fragments were preincubated for 15 min with the WCE before the addition of the DNA templates. Reactions were stopped after 45 min at 24°C , and the RNA runoff transcripts were purified and analyzed on 5% acrylamide-8.3 M urea gels as described previously (11). The pM17 plasmid was made by annealing the *EcoRI*-*HindIII* large (4.1-kilobase) fragment of pM15 (24) with the *EcoRI*-*SmaI* small (552-base-pair) fragment of pM8 (24) and a synthetic double-stranded oligonucleotide (sequence -124 to -86 of the MLP) with a double point mutation at -115 (G $\rightarrow$ A) and -113 (C $\rightarrow$ T). The oligonucleotide had a TCGA tetranucleotide at -86 to allow ligation with the *HindIII* restriction end of the pM15 fragment.

Preparation of labeled templates. DNA templates were ^{32}P 5' end labeled on one strand either upstream (RNA-noncoding strand) or downstream (RNA-coding strand) from the Ad2 MLP start site (21, 24). To label the noncoding strand, pM677 was digested by *SacII* at -245, dephosphorylated with calf intestinal phosphatase (Boehringer GmbH, Mannheim, Federal Republic of Germany), 5' end labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 polynucleotide kinase, and digested with *BamHI* at +33. The resulting *SacII*-*BamHI* (-245 to +33) DNA fragment was purified on a 6% acrylamide gel and eluted. To label the coding strand, pM677 and pM1 were linearized at the *BamHI* site at +33 and their 5' ends were dephosphorylated and ^{32}P phosphorylated, digested by *SacII* at -245, and purified as described above. The synthetic oligonucleotides were ^{32}P 5' end labeled on both strands with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 polynucleotide kinase and purified on a 6% acrylamide gel.

DNase I footprinting. DNase I footprinting consisted of a 10-min preincubation at 24°C in a 18- μl reaction volume containing variable amounts of the protein fraction, ~ 1 ng (10,000 cpm) of the 5'-end-labeled DNA fragment, 50 to 100 ng of unlabeled poly(dI-dC)-poly(dI-dC), and 4 mM MgCl_2 in buffer A. After the preincubation, 2 μl of DNase I (10 $\mu\text{g}/\text{ml}$) (Worthington Diagnostics, Freehold, N.J.) was added for 2 min and DNA digestion products were purified by phenol extraction, precipitated with ethanol, and analyzed on 8% acrylamide-8.3 M urea gels followed by autoradiography.

Gel retention assay. The gel retention assay (13) consisted of a preincubation step identical to that of the footprinting assay in a 10- μl reaction volume containing 100 to 250 ng of poly(dI-dC)-poly(dI-dC). After 15 min at 24°C , 1 μl of 87%

glycerol was added and the mixture was immediately electrophoresed at 30 mA on a 4.5% polyacrylamide gel (ratio of polyacrylamide-bisacrylamide, 80:1); the electrophoresis buffer was 6.7 mM Tris hydrochloride (pH 7.9)-3.3 mM sodium acetate-1 mM EDTA. After 2 h, the gel was dried and autoradiographed at -80°C .

Other techniques. Protein concentration was determined by the BCA protein assay of Pierce. Protein gel electrophoresis was done on sodium dodecyl sulfate-9% polyacrylamide gels, and staining was done by the silver staining method (41).

RESULTS

UEF binds to two sites in Ad2 IVa2-MLP intergenic region.

The improved purification procedure employed an affinity column containing a double-stranded DNA oligonucleotide with the sequence of the UE (from nucleotides -41 to -71) of the Ad2 MLP (see Materials and Methods and references 18 and 26). The UEF was eluted with a 1 M KCl wash from the DNA affinity column and was further purified on a hydroxylapatite column. The HAP0.12 fraction contained a single polypeptide of 45 kDa (HAP0.12, Fig. 1A, lane 1) which was found to bind specifically to the Ad2 MLP-UE. It protected the Ad2 MLP wild type (pM677) from DNase I digestion on the noncoding strand from -50 to -66 (Fig. 1B, lanes 2 to 4) and on the coding strand from -49 to -67 (lanes 6 to 8). The purified UEF was also able to bind a more distal region, closer to the IVa2 initiation site. This protection spanned from nucleotides -100 to -122 on the noncoding strand (lanes 2 to 4) and from nucleotides -98 to -120 on the coding strand (lanes 6 to 8). DNase I-hypersensitive sites were found at nucleotides A-121 and C-123 on the coding strand and at nucleotides T-98 and C-97 on the noncoding strand. We defined -98 as the limit of the UEF protection on the coding strand, since in some experiments we observed weak hypersensitive sites at nucleotides G-97 and C-95 (data not shown). Note that the second binding site (IVa2-UE) contains a palindromic sequence which shows some significant homology with the MLP-UE (see Fig. 1C for a summary of the footprint results).

To study whether or not the purified UEF was capable of recognizing the two binding sites independently, we performed two experiments. First, labeled double-stranded oligonucleotides (Fig. 1C) containing either the MLP-UE from nucleotides -41 to -71 (oligo-MLP) or the IVa2-UE from nucleotides -98 to -123 of the MLP start site (oligo-IVa2) were used in a gel retention assay. Addition of the purified UEF to either probe resulted in the formation of nucleoprotein complexes (Fig. 2A) with identical electrophoretic mobilities. Second, a double point mutation in the MLP-UE (at positions -60 and -62; pM1 mutant) which altered the binding of the UEF to the MLP-UE (25) did not affect the binding of the UEF to the IVa2-UE, as evidenced by DNase I footprinting (Fig. 1B, lanes 10 to 12). Taken together, these data demonstrate that binding of the UEF to one UE does not require binding to the other one.

UEF binds to both UEs with different affinities. According to gel retention assays, formation of the oligo-IVa2-UEF complex required two to three times more UEF than formation of the oligo-MLP-UEF complex (Fig. 2A, compare lanes 4 and 12). Furthermore, in the DNase I footprinting experiments, complete protection of the IVa2-UE required at least five times more UEF than that of the MLP-UE (Fig. 1B, compare lanes 6 and 8; see also Fig. 2D, lane 2, in which the protection of the IVa2-UE is more complete). To further

FIG. 1. (A) Sodium dodecyl sulfate-polyacrylamide gel analysis of the purified fraction of the UEF. Lanes: 1, 40 μ l of the HAP0.12 fraction; 2, molecular weight markers ($\times 10^3$). The arrow shows the 45-kDa polypeptide which correlates with the UEF activity. (B) DNase I footprinting experiments with the noncoding (lanes 1 to 4) or the coding (lanes 5 to 8) strand of the wild-type Ad2 MLP recombinant pM677 or the coding strand (lanes 9 to 12) of the upstream sequence double point mutant pM1. The 32 P-5'-end-labeled DNAs were prepared as described previously (24) (see also Materials and Methods). Reactions were done in the absence (-) or presence of 2, 5, or 10 ng of the DNA-affinity-purified UEF. The locations of the protected UE regions and of the IVa2 promoter and MLP initiation sites are indicated. (C) Summary of DNase I footprinting on the upstream sequences of the Ad2 MLP and IVa2 promoter. The nucleotide sequences of the RNA noncoding (NC) and coding (C) strands of the MLP upstream regions are shown. The regions protected from DNase I digestion by factor binding are shown by brackets, and DNase I-hypersensitive sites are indicated by arrows. The bases pointed out by asterisks denote the homology between the two protected regions. The regions exhibiting a dyad symmetry are indicated by arrows pointing in opposite directions. The numbers above the sequence show the positions with respect to the MLP initiation site. The positions of the same sequences with respect to the IVa2 initiation site are given by the numbers in parentheses. The sequences encompassed by the synthetic oligonucleotides (oligo-MLP and oligo-IVa2) are also shown by brackets.

study the affinity of the UEF for the two binding sites (IVa2-UE and MLP-UE), we used a 32 P-end-labeled DNA probe (pM677 from -245 to +33; see Materials and Methods) in a gel retention assay. When increasing amounts of the UEF fraction were added to the labeled DNA, we first observed the formation of nucleoprotein complex I (Fig. 2B, lanes 2 and 3), which progressively disappeared with the concomitant appearance of nucleoprotein complex II, which had a lower electrophoretic mobility (lanes 4 and 5). When the UEF was in excess, only nucleoprotein complex II was present (lane 6). There was an additional nucleoprotein complex which migrated in the vicinity of nucleoprotein complex I and could result from a partially degraded form of the UEF. When only nucleoprotein complex I was apparent (Fig. 2B, lane 2), only the MLP-UE was protected as observed in the DNase I footprinting pattern (Fig. 1B, lanes 2 and 6). At higher concentrations of UEF, the appearance of complex II (Fig. 2B, lane 4) paralleled the protection of the two UEs (Fig. 1A, lanes 4 and 8).

To further characterize nucleoprotein complexes I and II, we used a competition analysis in which the radiolabeled wild-type DNA probe (pM677) was preincubated with increasing amounts of either oligo-MLP or oligo-IVa2. Thus, the ability of each oligonucleotide to compete for both nucleoprotein complexes was monitored (Fig. 2C). The addition of increasing amounts of oligo-MLP resulted in the disappearance of nucleoprotein complex II, followed by a decrease in nucleoprotein complex I (Fig. 2C, lanes 2 to 6). When oligo-IVa2 was used as the competitor, the decrease of complex II was concomitant with an increase in complex I (lanes 7 to 10). This intermediate increase in complex I was probably the result of a preferential competition of the binding at the weaker site (IVa2-UE) which thus transformed complex II to complex I, before this latter complex was also competed out by higher amount of competitor. Higher amounts (12 ng) of oligo-IVa2 competitor led to the disappearance of complex I (Fig. 2C, lane 11). The addition of either 40 ng of poly(dI-dC)-poly(dI-dC) or 40 ng of a nonspecific oligonucleotide (from -70 to -90 of the Ad2 MLP) did not compete for the formation of nucleoprotein complexes (lanes 12 and 13, respectively), indicating that the complexes are sequence specific. The DNase I footprinting competition experiments (Fig. 2D) paralleled the results obtained with the gel retention assay. Indeed, a deprotection of the IVa2-UE required more oligo-IVa2 (50 ng) than oligo-MLP (5 ng) competitors (Fig. 2D, compare lanes 6 and 11). Similarly, a deprotection of the MLP-UE was obtained with 300 ng of oligo-IVa2 and 20 ng of oligo-MLP competitors (compare lanes 8 and 13, respectively). As a control, 300 ng of poly(dI-dC) (lane 3) or 300 ng of the nonspecific oligonucleotide (used in the gel retention assay; Fig. 2C; other data not shown) did not deprotect either of the UEs.

In conclusion, our *in vitro* experiments showed that the UEF binds first to the MLP-UE with a high affinity and then to the IVa2-UE with a lower affinity; nucleoprotein complex I corresponds to the binding of one UEF molecule to the MLP-UE, whereas nucleoprotein complex II corresponds to the occupancy of each UE by one UEF molecule (see also reference 19). This was further confirmed by DNase I footprinting of the isolated nucleoprotein complexes I and II (data not shown). Note that, as a consequence of the highest affinity for the MLP-UE, the binding at IVa2-UE was detected (by the appearance of complex II) only after complete retention of the free DNA in complex I; thus, the appearance of complex II was correlated with the absence of free DNA on the autoradiogram.

FIG. 2. Interaction of the UEF with the MLP- and IVa2-UE. (A) Gel retention assay with UEF and either synthetic double-stranded oligonucleotide. Increasing amounts of purified UEF, as indicated at the top of the figure, were incubated with either ³²P-labeled oligo-MLP (lanes 1 to 7) or ³²P-labeled oligo-IVa2 (lanes 8 to 14), electrophoresed on a 4.5% polyacrylamide gel, and autoradiographed. The positions of free and protein-bound probes are indicated by the arrows. (B) Nucleoprotein complex formation between the UEF and the ³²P-labeled *SacII-BamHI* fragment of pM677 encompassing both UEs. Increasing amounts of UEF, as indicated (in nanograms) at the top of the figure, were incubated with the ³²P-end-labeled *SacII-BamHI* fragment of pM677. Free and protein-bound DNAs (complexes I and II) were separated by electrophoresis. (C) Competition of the nucleoprotein complexes in a gel retention assay. Samples of 10 ng of UEF and 100 ng of poly(dI-dC)-poly(dI-dC) were incubated with the ³²P-end-labeled *SacII-BamHI* fragment of pM677 and increasing amounts (in nanograms) of oligo-MLP (lanes 3 to 6) or oligo-IVa2 (lanes 7 to 11), 40 ng of poly(dI-dC)-poly(dI-dC) (lane 12), or 40 ng of the nonspecific double-stranded oligonucleotide (ns) from -70 to -90 with respect to the major late initiation site (lane 13). Complexes I and II (arrows) were separated by electrophoresis. Lane 1 contains no protein. (D) Competition of the footprint on the coding strand of MLP by either oligonucleotide. A 10-ng portion of UEF was incubated with the ³²P-5'-end-labeled *SacII-BamHI* fragment of pM677 and various amounts (as indicated in nanograms at the top of the figure) of either oligo-IVa2 (lanes 4 to 8) or oligo-MLP (lanes 10 to 15). Lane 1 contains no protein. Lane 2 contains no competitor. Lane 3 contains 300 ng of poly(dI-dC)-poly(dI-dC) as a nonspecific competitor. In addition, a constant amount (50 ng) of poly(dI-dC)-poly(dI-dC) was also present in each reaction mixture. Lane 16 contains the size markers. The locations of the protected UE regions and of the IVa2 and MLP initiation sites are indicated on the left side of the figure.

UEF stimulates transcription of both IVa2 and MLP. It was previously demonstrated that binding of the UEF to the MLP-UE stimulates transcription from the MLP. A major problem that we (26) and others (10, 36) encountered, however, was an apparent loss of transcriptional activity with increasing purification of the UEF. Indeed, a loss of catalytic activity by modification of the functional domain could occur during purification, independent of any altera-

tion of the DNA-binding domain. This observation and the fact that transcription from the IVa2 promoter with our reconstituted system (26) is extremely weak (IVa2 lacks a TATA box) obliged us to measure transcriptional activity of both promoters in a WCE. We first estimated the influence of each proximal UE on the transcription of either the MLP or IVa2 promoter. A fixed amount of WCE which contained the four general transcription factors and RNA polymerase B

FIG. 3. Structure of the recombinant DNA used in in vitro transcription assays. The DNA fragments containing various lengths of Ad2 MLP and/or IVa2 promoter are shown. Thin lines represent adenovirus DNA; wavy lines represent pBR322 DNA; dotted lines represent deleted DNA. The major late and IVa2 initiation sites are represented by arrows. Filled triangles represent the wild-type MLP TATA box. Striped and open boxes represent wild-type and mutated UEF-binding sites, respectively. The proximal domain of the IVa2 promoter (–38 to –49 with respect to the IVa2 initiation site) is represented by a filled box.

(26) in addition to the UEF was preincubated with either the oligo-MLP or the oligo-IVa2 competitor before addition of the DNA template. The pM97 *EcoRI-SalI* and pM15 *EcoRI-HindIII* fragments, described in Fig. 3, were used for transcription from the MLP and IVa2 promoter, respectively. Each template contained only its proximal UE 5' to the RNA start site and gave a single runoff transcript of the expected size for the MLP (309 nucleotides) and the IVa2 promoter (466 nucleotides) (Fig. 4A and B, respectively, lanes 1). The addition of increasing amounts of the oligo-MLP fragment to the preincubation mixture resulted in a decrease in transcription from both the MLP (pM97 *EcoRI-SalI*; Fig. 4A, lanes 2 to 4) and the IVa2 promoter (pM15 *EcoRI-HindIII*; Fig. 4B, lanes 2 to 4). To decrease transcription from either promoter, higher amounts of the oligo-IVa2 fragment were necessary (Fig. 4A and B, lanes 5 to 7). Densitometric scanning of autoradiograms similar to those shown in Fig. 4A and B indicated clearly that when using both competitors, MLP and IVa2 transcription decreased to a basal level (Fig. 4C; and see below). We had previously defined the basal level of transcription as the rate of transcription obtained with a minimal promoter (from –34 to +33 for the MLP; see reference 26) in the absence of any UE. Preincubation of the WCE either with poly(dI-dC)-poly(dI-dC) (Fig. 4A and B, lanes 8) or with a nonspecific oligonucleotide (Fig. 4C) had no effect on the transcription efficiency of either the MLP or IVa2 promoter. Furthermore, a double point mutation in the IVa2-UE (similar to the double point mutation in the MLP-UE at –60 and –62 which was shown to prevent the binding of UEF [24, 25]) produced a three- to fourfold decrease of IVa2 transcription (Fig. 5B, compare lanes 1 and 3). However, these mutations did not abolish transcription of the IVa2 promoter, indicating that the IVa2, as equally observed with the MLP (24), may work without the IVa2-UE (see also Fig. 5B). Note that the pM8 *EcoRI-SmaI* template, which has no IVa2-UE, was also able to be transcribed at a similar level as the point-mutated template (Fig. 5B, lane 4). Taken together, all these results demonstrated that the stimulation of the MLP or IVa2 promoter is

mediated by the binding of the UEF to their respective UE binding sites (MLP-UE and IVa2-UE, respectively).

We then investigated the influence of the distal UE on transcription from both promoters. Three MLP templates differing in their upstream sequences (Fig. 3) were tested in an in vitro transcription system. The pM8 *SmaI-SalI* template (which contains the two UEs, IVa2-UE and MLP-UE) was slightly more efficiently transcribed than either the pM97 *EcoRI-SalI* template (which has only the MLP-UE) or the pM34 *EcoRI-SalI* template (which has no UE) (Fig. 5A, upper panel). The IVa2-UE had a weak but reproducible stimulatory effect on MLP transcription; indeed, the pM8 template showed around 15% higher transcription than the pM97 template. In another set of experiments, we also studied the influence of the distal MLP-UE element on IVa2 gene transcription (Fig. 5A, lower panel). We used either the pM677 template, which contains both the MLP-UE and the IVa2-UE (pM677 *EcoRI-SalI* template; Fig. 3) or the pM1 template, which has a double point mutation in the MLP-UE at positions –60 and –62 (preventing the binding of UEF to MLP-UE [24] [Fig. 1B]) and has the intact IVa2-UE. In this case, mutation of the distal UE resulted in a slight and reproducible decrease (10%) of the transcription efficiency of the IVa2 promoter (Fig. 5A, lower panel). In conclusion, for both the MLP and IVa2 promoter, the distal UE had a much weaker transcription-stimulatory effect than the proximal one.

DISCUSSION

We showed by gel retention assays and DNase I footprinting that the Ad2 major late UEF purified from HeLa cells is able to recognize two UEs located in the MLP-IVa2 intergenic region: the MLP-UE located between nucleotides –49 and –67 and the IVa2-UE which spans from nucleotide –98 to –122. Comparison of the UEs reveals a sequence homology with a conserved palindromic structure in which 7 of 10 nucleotides are identical; this is also found in several genes of higher eucaryotes (6, 9, 12, 27–29) as well as in lower eucaryotes (2, 3, 39).

FIG. 4. In vitro transcription competition of MLP and IVa2 promoter with oligo-MLP or oligo-IVa2. The templates used for MLP and IVa2 transcription were the *EcoRI-SalI* fragment of pM97 and the *EcoRI-HindIII* fragment of pM15, respectively (Fig. 3). Increasing amounts (see at the top of the figure) of either oligonucleotide were preincubated with the HeLa WCE before addition of the DNA templates as described in Materials and Methods. (A and B) Lanes 1 contain no competitor. Lanes 8 contain 50 ng of poly(dI-dC)-poly(dI-dC) as a nonspecific competitor. The arrows indicate either MLP transcription (309 nucleotides [A]) or IVa2 transcription (466 nucleotides [B]). (C) Densitometer quantitation of the data shown in panels A and B and of data not shown that were obtained in several competition experiments. ns, Nonspecific oligonucleotide covering the region -70 to -90 of the MLP.

The two UEs have different affinities toward the UEF. As demonstrated by gel retention and footprinting competition experiments, the UEF binds to the IVa2-UE with a lower affinity than to the MLP-UE. Indeed, the central CAC/GTG palindrome in the MLP-UE is critical for the UEF binding. A deviation in the first G of the palindrome for IVa2 (this

FIG. 5. Requirement of the MLP-UE and IVa2-UE for efficient MLP or IVa2 in vitro transcription. The wild-type Ad2 MLP recombinant pM677 and the series of upstream deletions (Fig. 3) were transcribed with a WCE as described in Materials and Methods. (A) The level of in vitro transcription (in arbitrary units) of the MLP and IVa2 promoter is shown in the upper and lower panels, respectively, as a function of the amount of DNA template (in picomoles). In the upper panel, the templates used were pM8 *SmaI-SalI* (○), pM97 *EcoRI-SalI* (●), pM34 *EcoRI-SalI* (△), and pM1 *EcoRI-SalI* (▲). In the lower panel, the templates used contained both promoters; both were wild type (pM677 *EcoRI-SalI* [■]), or one was mutated in the MLP-UE (pM1 *EcoRI-SalI* [□]), or one was mutated in the IVa2-UE (pM17 *EcoRI-SalI* [×]). The values correspond to an average of five different experiments with two different preparations of DNA templates. (B) A mutation in the IVa2-UE decreases in vitro transcription of the IVa2 promoter. Lanes: 1, pM15 *EcoRI-HindIII* (wild type); 2, same as in lane 1 with α-amanitin; 3, pM17 *EcoRI-HindIII* (double point mutation in the IVa2-UE); 4, pM8 *EcoRI-SmaI* (deletion of the IVa2-UE); size markers (in nucleotides) are as indicated. The arrow indicates the IVa2 transcript (466 nucleotides).

report), in the first C for the MLP-UE (24), or in the second C for human U1 gene (14) led to a decrease in the affinity of the UEF for its binding site. In addition, there is a correlation between the affinity of the UEF for its binding site and its stimulatory effect toward the proximal promoter (this report; see also below for discussion). Indeed, the binding of the UEF, which is stronger on the MLP-UE than on the IVa2-UE, results in a higher transcription stimulation of the MLP than of the IVa2 promoter.

Although the two UEs are close to each other, the binding of the UEF to the two sites does not seem to be cooperative. First, nucleoprotein complexes can be formed independently on either site (Fig. 1B and 2A). Second, the two sites are filled sequentially, and binding to the low-affinity site is only observed when the high-affinity site is already filled (Fig. 2B).

The role of these UEs in the transcription efficiency of both the MLP and IVa2 promoter was analyzed by *in vitro* transcription. First, we demonstrated that the IVa2 promoter as well as the MLP may work in the absence of their respective proximal UEs (Fig. 4) (26). This was equally observed for the adenovirus MLP (5), the mouse metallothionein promoter (6), and the rat γ fibrinogen promoter (9). Since the UE and more likely its cognate binding factor are not absolutely required for basal transcription, we conclude that the UEF is a gene-specific factor rather than a general transcription factor. However, the IVa2 promoter is two- to threefold stimulated by its IVa2-UE, as also observed for the MLP (Fig. 4 and 5) (24, 25).

We also found that either promoter is stimulated by the distal UE, although to a much lower extent than by the proximal element (Fig. 5). This low but reproducible degree of UEF responsiveness once bound to the distal promoter, also noted in the γ fibrinogen promoter (9), may reflect the location (distance effect) of the UEF-binding site from the initiation site. Indeed, insertion of spacing sequences between the TATA box and the Sp1-binding site in the simian virus 40 early promoter (38) or between the TATA box and the MLP-UE (20) results in a decrease of transcription efficiency of the UEF. This also explains the weak stimulatory effect of the distal UE on transcription efficiency of both promoters, even when the proximal UE is downmutated. Chodosh et al. (9) also suggest that the lower degree of UEF responsiveness reflects the more distal location of the UEF-binding site in the γ fibrinogene promoter.

Previous studies have shown that the UEF (37) as well as ATF (17) and GAL4 (16) stabilized the binding of the BTF1 (the TATA box-recognizing factor) on the TATA box. On the other hand, the IVa2 as well as the 3-hydroxy-3-methylglutaryl CoA reductase (33) and the mouse thymidylate synthase (12) genes, which have a UEF-binding site, lack a consensus TATA box. Thus, it is possible that besides UEF-BTF1 protein interaction, other interactions between the UEF and the general transcription machinery (e.g., RNA polymerase B, STF, BTF2 and BTF3, etc.) are probably important for positioning and/or activating gene transcription.

There are slight discrepancies between our results and those of others. Previously, using crude preparations of UEF, binding of the UEF has been detected only to the MLP-UE (32), and consequently, it has been believed that IVa2 transcription is under the control of the MLP-UE. To observe the IVa2-UE binding site, we were obliged to use highly purified fractions of UEF; cruder UEF preparations did not allow us to observe the binding at IVa2-UE, certainly because this site has a lower affinity for the UEF.

It is tempting to speculate that the relative occupancy of both UEs (present in the MLP-IVa2 intergenic region) by the UEF influences the transcription rate from both the MLP and IVa2 promoter during the viral cycle. However, the dramatic increase of both IVa2 and major late gene transcription rates during the late phase of viral development suggests that the threefold stimulation of IVa2 transcription by the IVa2-UE observed in our *in vitro* system is not sufficient to account for this effect. We also cannot exclude that the UEF could be the target of another (inducible) factor required to mediate, in a synergistic manner, a high and selective increase of the transcription from both promoters.

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