Journal of Chromatography, 510 (1990) 243–250
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 424

Purification of the upstream element factor of the Adenovirus-2 major late promoter from HeLa and yeast by sequence-specific DNA affinity chromatography

VINCENT MONCOLLIN, MATTHIEU GERARD and JEAN-MARC EGLY*

Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, 11 Rue Humann, 67085 Strasbourg Cédex (France)

ABSTRACT

The purification to homogeneity of the Adenovirus-2 major late promoter (MLP) upstream element factor (UEF), a sequence specific transcription factor, which binds to upstream elements of various class B (II) genes, is reported. The protein was purified from HeLa cells and also from the yeast *Saccharomyces cerevisiae*, by using sequence-specific DNA affinity chromatography. The human (UEFh, 45 000 dalton) and the yeast (UEFy, 60 000 dalton) proteins protect the same sequences over the MLP-IVa2 intergenic region: the MLP-UE (from nucleotide −49 to −67) and the IVa2-UE (from nucleotide −98 to −122 relative to the MLP initiation site). Both proteins have a higher affinity for the MLP-UE than for the IVa2-UE.

INTRODUCTION

Transcription of protein coding genes requires the presence of distinct promoter elements in the DNA template: (1) the initiation site and the TATA-box, a highly conserved AT-rich region located 30 base pairs (bp) and 40–110 bp upstream of the transcription start site in higher and lower eukaryotes, respectively, and (2) the upstream elements which are located in the −40 to −110 region upstream from the start site (for a review, see ref. 1). *In vivo* and *in vitro* studies have shown that these promoter elements are the target of various transcription factors. While the TATA-box interacts with the TATA-box recognizing factor BTF1[2], also called TFIID[3], both the upstream elements and enhancers bind sequence specific factors (*e.g.*, Sp1, HSTF, CBP) which have some regulatory function[1].

The HeLa upstream element factor (UEF) of the Adenovirus-2-major late promoter (Ad2 MLP) had been shown to stimulate Ad2 MLP transcription both *in vivo* and *in vitro* through the binding at the MLP upstream element (MPL-UE located between positions −49 and −67 of the Ad2 MLP)[4]. Further it has also been suggested that stimulation of transcription by the sequence-specific upstream factors occurs

0021-9673/90/$03.50

through interaction with the TATA-box recognizing factor[5,6]. On the other hand, recent studies have demonstrated that the yeast TATA-box factor (BTF1y) can accurately initiate *in vitro* transcription when added to the HeLa system lacking mammalian BTF1[7,8]. These results suggest that protein–protein and DNA–protein interactions, involved in the initiation of transcription of protein coding genes, have been conserved during evolution. In a first step to understanding such mechanisms, it was necessary to identify and purify the various factors involved in the transcription machinery. We report here the purification of the UEF factor from both HeLa (UEFh) and yeast (UEFy) by using classical chromatographic techniques and also a sequence-specific DNA affinity column. Both proteins recognized the same upstream elements: the MLP-UE binding site (from −49 to −67) and the IVa2-UE binding site (from −98 to −122).

EXPERIMENTAL

Purification of the UEF from HeLa or yeast

The UEFh was purified from a HeLa whole cell extract (WCE) and the three first steps of the purification procedure (heparin–Ultrogel, DEAE-5PW, SP-5PW) were as previously described[9,10]. The SP0.35 fraction was dialysed in buffer A [50 m*M* Tris–HCl (pH 7.9)–50 m*M* KCl–8.7% glycerol–0.1 m*M* EDTA–0.5 m*M* dithiothreitol (DTT)] and incubated for 15 min at 4°C with 100 μg/ml of poly(dI–dC) (dI–dC) (Pharmacia, Uppsala, Sweden) and 4 m*M* $MgCl_2$, before being loaded onto a 1-ml sequence-specific DNA affinity column made as follows: the two 35-mer synthetic oligonucleotide strands (nucleotides −41 to −71 with respect to the Ad2 MLP start site with a GATC tetramer added at the 5′-terminus) were hybridized and ligated with T4 DNA ligase. The polymers were fixed onto a Sepharose CL-4B resin (Pharmacia) preactivated with cyanogen bromide[11]. The column was then washed successively with ten column volumes of buffer B [50 m*M* Tris–HCl (pH 7.9)–50 m*M* KCl–17.4% glycerol–5 m*M* $MgCl_2$–0.1% Nonidet P40 (NP40)–1 m*M* DTT] and five column volumes of buffer B containing 0.3 *M* KCl. The UEFh was then eluted with ten column volumes of buffer B containing 1 *M* KCl. The active 1 *M* KCl fractions (5 ml) were dialysed against buffer C [10 m*M* potassium phosphate (pH 7)–0.01 m*M* $CaCl_2$–8.7% glycerol–0.5 m*M* DTT] and loaded on a hydroxyapatite column (500 μl) (BDH, Poole, U.K.) pre-equilibrated in buffer C. The UEFh was eluted with 0.12 *M* potassium phosphate in buffer C (five column volumes). The fractions were dialysed against buffer D [50 m*M* Tris–HCl (pH7.9)– 50 m*M* KCl–25% glycerol–0.1 m*M* EDTA–0.5 m*M* DTT] and stored at −80°C. The UEFy was purified as follows. An extract of the yeast *Saccharomyces cerevisiae* S-100 (5 ml; 37 mg/ml) prepared as previously described [12] was dialysed for 12 h against buffer E [50 m*M* Tris–HCl (pH 7.9)–50 m*M* KCl–17.4% glycerol–2 m*M* $MgCl_2$–0.5 m*M* DTT–0.1 m*M* PMSF] and incubated for 15 min at 4°C with 100 μg /ml of poly(dI–dC) (Pharmacia) and 0.1% NP-40 and then for 15 min at 4°C with 1 ml of the sequence-specific DNA affinity resin. The resin was then packed in a Pasteur pipette and washed with ten column volumes of buffer F [50 m*M* Tris–HCl (pH 7.9)–50 m*M* KCl–17.4% glycerol–2 m*M* $MgCl_2$–0.1% NP-40–1 m*M* DTT), five column volumes of buffer F containing 0.2 *M* KCl and five column volumes of buffer F containing 1 *M* KCl. After dialysis against buffer F, the 1 *M* KCl fraction was incubated for 15 min at

4°C with 50 μg/ml of poly(dI–dC)(dI–dC) and reapplied to the DNA affinity column. The 1 *M* KCl eluate from this second affinity column (*ca.* 2 μg/ml) was dialysed against buffer D and stored at −80°C. Purified fractions were analysed electrophoretically on 9% sodium dodecyl sulphate—polyacrylamide gels (SDS-PAGE).

DNAse I footprinting and gel retention assays

For labelling the non-coding strand, pM677 was digested by the restriction enzyme SacII at −245, dephosphorylated with calf intestinal phosphatase (Boehringer), 5′-end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase, then digested with the restriction enzyme BamHI. The resulting SacII–BamHI (−245/+33) DNA fragment was purified on a 6% acrylamide gel. For labelling the coding strand, pM677 was linearized at the BamHI site at +33, ^{32}P-phosphorylated, digested by SacII at −245 and the fragment was purified as described above.

DNAse I footprinting reactions consisted of a 10-min preincubation at 24°C in an 18-μl reaction volume containing various amounts of the protein fraction, *ca.* 1 ng (10 000 cpm) of the 5′-end-labelled DNA fragment, 50 ng of poly(dI–dC)(dI–dC) and 4 m*M* $MgCl_2$ in buffer A. After the preincubation, 2 μl of DNAse I (10 μg/ml) (Worthington, U.K.) was added for 2 min and DNA digestion products were analysed on 8% acrylamide–8.3 *M* urea gels, followed by autoradiography.

The gel retention assay[13] consisted of a 15-min incubation step at 24°C identical with that in the footprinting assay in a 10-μl reaction volume containing 100–250 ng of poly(dI–dC)(dI–dC) (Pharmacia). A 1-μl volume of 87% glycerol was added and the mixture was electrophoresed immediately on a 4.5% polyacrylamide gel (polyacrylamide–bisacrylamide, 80:1). The electrophoresis buffer was 6.7 m*M* Tris–HCl (pH 7.9)–3.3 m*M* sodium acetate–1 m*M* EDTA. The gel was dried and autoradiographed.

RESULTS

It has generally been very difficult to purify proteins involved in the regulation of transcription to homogeneity because they usually constitute less than 0.001% of the total cellular protein. That is why specific-DNA-affinity chromatography was an attractive separation procedure for increasing the DNA-binding factor recovery[14]. To purify both UEF factors, we designed a DNA-affinity column containing a polymer of the Ad2 MLP upstream element (from nucleotide −41 to −71) (see ref. 15 and Experimental). The DNA-binding activity was detected during the purification procedure by a gel retention assay. This assay is based on the slower migration of a DNA–protein complex compared with the free DNA on a non-denaturing polyacrylamide gel[13].

Purification of the HeLa UEF

The purification scheme for the HeLa UEF is summarized in Fig. 1A and Table I. HeLa whole cell extract was applied successively to heparin–Ultrogel, DEAE-5PW and SP-5PW columns as previously described[10]. The critical step of the procedure was the use of a sequence-specific DNA affinity column prepared as described under Experimental. The SP0.35 fraction, which contains the UEF, was preincubated with a non-specific DNA competitor (polydI–dC/dI–dC) in order to prevent the interactions

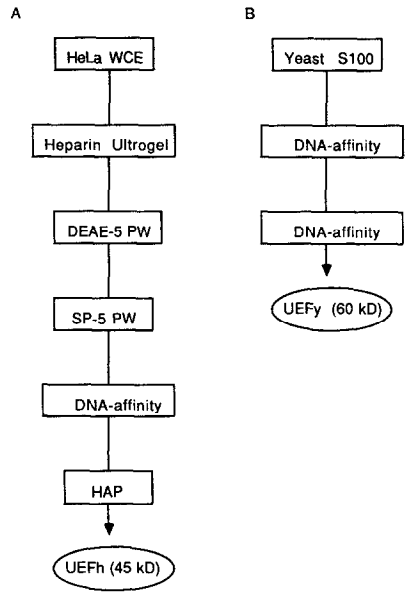

Fig. 1. Purification scheme of Ad2 MLP-UEF from (A) HeLa or (B) yeast. kD = Kilodalton.

of non-specific DNA binding proteins on the MLP-UE site, and loaded onto the affinity column. The 1 *M* KCl eluate contains two polypeptides of 40 000 and 45 000 dalton (data not shown), which co-migrates with the binding activity to the MLP-UE. These two polypeptides were separated by chromatography on a hydroxyapatite column; as shown by SDS-PAGE, the HAP0.12 fraction contained a single polypeptide of 45 000 dalton (Fig. 2A, lane 1). To provide additional evidence that the 45 000-dalton polypeptide corresponds to the Ad2 MLP-UEF, the purified polypeptide was

TABLE I

SUMMARY OF UEFh AND UEFy PURIFICATION

	Step	*Volume (ml)*	*Protein (mg/ml)*	*Total protein (mg)*	*Units*[a]	*Specific activity (units/mg)*	*Purification (factor)*	*Yield (%)*
UEFh	HeLa WCE	230	7	1610	460 000	285	–	–
	Heparin–Ultrogel	150	0.7	105	300 000	2860	10	65
	DEAE-5 PW	22	0.45	9.9	110 000	11 000	38	24
	SP-5 PW	10	0.40	4	66 000	16 500	58	14
	DNA-affinity	5	0.001	0.005	40 000	$8 \cdot 10^6$	28 000	8.7
	Hydroxyapatite	0.5	0.005	0.0025	20 000	$8 \cdot 10^6$	28 000	4.3
UEFy	Yeast S-100	5	37	185	50 000	270	–	–
	DNA-affinity 1	4	0.02	0.1	33 000	330 000	1200	66
	DNA-affinity 2	4	0.002	0.008	26 600	$3.3 \cdot 10^6$	12 000	53

[a] One unit is defined as the amount of protein that produces retention of 0.05 ng of DNA fragment in the gel retention assay.

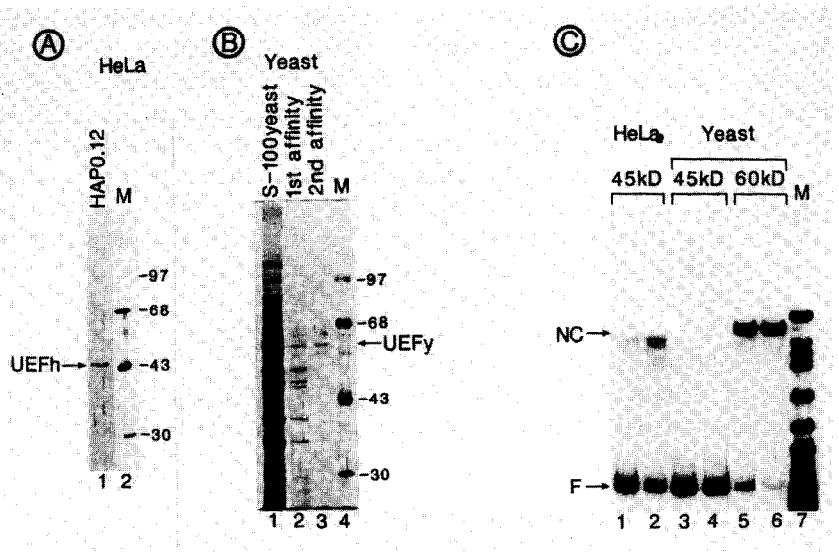

Fig. 2. (A) SDS-PAGE of purified UEFh. Lanes: 1 = 50 μl of the HAP0.12 fraction; 2 = molecular weight markers. The arrow indicates the 45-kDa polypeptide (UEFh). (B) SDS-PAGE of purification steps of the UEFy. Lanes: 1 = 0.5 μl of yeast S-100 extract; 2 and 3 = 100 μl of the 1 *M* KCl eluate of the first and second DNA affinity columns, respectively; 4 = molecular weight markers. The arrow indicates the 60-kDa polypeptide (UEFy). (C) Nucleoprotein complexes formed between the polypeptides renatured after elution from the SDS gel, and the ^{32}P-labelled SacII–BamHI fragment of pM677. Binding reactions included 100 ng of poly(dI–dC)(dI–dC), 0.1 ng of the labelled DNA and either 2–4 μl of the renatured 45 000-dalton HeLa polypeptide (lanes 1 and 2), 2–4 μl of the renatured yeast 45 000-dalton region (lanes 3 and 4) or 2–4 μl of the renatured 60 000-dalton yeast polypeptide (lanes 5 and 6). Lane 7 = size markers. NC and F indicate the nucleoprotein complexes and the free DNA, respectively.

eluted and renatured from an SDS polyacrylamide gel[16]. After renaturation, this polypeptide was able to form a specific complex with a labelled DNA containing the MLP-UE site, as detected by the gel retention assay (Fig. 2C, lanes 1 and 2). Starting from $3 \cdot 10^{10}$ HeLa cells (40 l of culture), *ca.* 2.5 μg of purified UEFh were recovered at the end of the purification (overall purification 28 000-fold; Table I). It should be noted that both polypeptides isolated by the affinity column possess the DNA-binding activity, which explains why the hydroxyapatite used to fractionate this two polypeptides did not increase the specific activity (see Table I).

Purification of the yeast UEF

The purification scheme for the UEFy is summarized Fig. 1B and Table I. A yeast S-100 extract was applied to the previously described DNA affinity matrix. After two passes on the affinity column the 1 *M* KCl eluate fraction contained a single polypeptide of 60 000 dalton (Fig. 2B, lane 3). After SDS-PAGE and renaturation, only the fraction containing the 60 000-dalton polypeptide was able to form a specific nucleoprotein complex with the labelled DNA containing the MLP-UE site (Fig. 2C, lanes 3–6). Further, a competition assay was used to check the specificity of the DNA-binding complex. The competition DNA-binding assay involved the sequential

addition of two different DNA fragments and was based on the assumption that a DNA-binding protein could bind more tightly to DNA fragments containing the wild-type UE sequence than to mutated or non-specific DNA fragments. The presence of a specific nucleoprotein complex would then be strongly reduced or eliminated, provided that the unlabelled wild-type competitor fragment was present in excess and its interaction with the factor was sufficiently stable. Preincubation of either UEFh or UEFy with a DNA fragment containing the wild-type MLP-UE prevents the formation of nucleoprotein complexes, thus demonstrating that the nucleoprotein complexes were specific for the MLP-UE. When non-specific (polydI–dC/dI–dC) or the mutated MLP-UE (which does not bind the UEF[4]) were used the formation of the complexes was not altered (data not shown). By centrifugation on a glycerol gradient, the DNA-binding activity of the UEFh and UEFy were detected in fractions corresponding to molecular weights of 45 000 and 60 000 dalton, respectively, in agreement with the observed molecular weight derived from SDS-PAGE[15,17] (data not shown).

Yeast and human UEF interact with identical DNA sequences

In order to delineate precisely the DNA region that interacts with the UEFh and UEFy, DNAse I footprint experiments were performed. On the coding strand, both the purified UEFh (Fig. 3, lane 2) and the purified UEFy (Fig. 3, lane 4) protected the MLP-UE from nucleotide −49 to −67 (with a strong DNAse I hypersensitive site at −69) and the IVa2-UE from nucleotide −98 to −120. On the non-coding

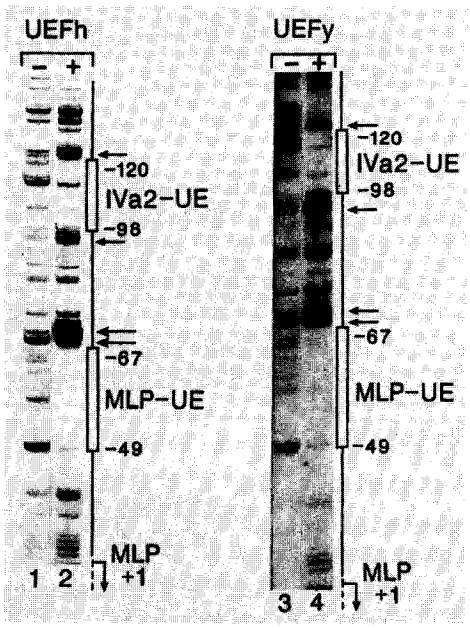

Fig. 3. DNAse I footprint on the coding strand of the MLP-IVa2 intergenic region. The ^{32}P-labelled DNA fragment (1 ng) was incubated with either UEFh (lane 2) or UEFy (lane 4) or in the absence of protein (lanes 1 and 3). The position of the MLP-UE, IVa2-UE and MLP initiation site are indicated. The arrows indicate the hypersensitives sites.

strand, the MLP-UE was protected from nucleotide −50 to −66 (MLP-UE) and from nucleotide −100 to −122 (IVa2-UE) (not shown).

The UEFy has affinities toward the two binding sites similar to those of the UEFh; they bind with a higher affinity to the MLP-UE ($K_d = 10^{-11}$) than to the IVa2-UE ($K_d = 10^{-10}$) (data not shown). Moreover, no cooperativity was apparent in the simultaneous binding of UEFy to these two sites (see also refs. 15 and 18).

DISCUSSION

By the use of a sequence-specific DNA affinity column, we purified to homogeneity two related proteins from HeLa (UEFh) and yeast (UEFy). The affinity chromatographic step improved the purification process, especially with the yeast protein; indeed, starting with the crude yeast extract, the purified UEFy was obtained by two passes over the affinity column with a relatively high recovery (50%) compared with the UEFh purification process (see Table I). This procedure was efficient both in time and in yield of protein, and the column was used more than ten times without a decrease in the capacity. In addition, we demonstrated that both UEFh and UEFy recognized the MLP-UE and IVa2-UE with identical DNAse I footprinting patterns, although these two proteins have different molecular weights. The similarity in the DNA-binding properties raises the possibility that the UEFy possesses the same transcription stimulatory property as the UEFh. We therefore tested the stimulatory effect of the UEFy in various *in vitro* transcription systems. Using the complete mammalian basic system (BTF1, BTF2, BTF3, STF, RNA polymerase B)[10], we were not able to detect a stimulation of *in vitro* transcription from the Ad2 MLP by the purified UEFy. It has been suggested that protein–protein interaction between the UEF and the TATA-box factor (BTF1) could be a prerequisite for transcription stimulation by the UEF. We therefore replaced mammalian BTF1 by its yeast counterpart (BTF1y) to allow protein–protein interaction between these two yeast proteins. However, this exchange did not elicit stimulation of transcription by addition of UEFy. There are several possibilities for this lack of stimulation in our previously designed transcription system. First, because of its higher molecular weight (60 000 *versus* 45 000 dalton for the UEFh), UEFy is not able to interact with the mammalian transcription machinery and may require a complete yeast transcription system or an additionnal yeast factor to stimulate Ad2 MLP *in vitro* transcription. It is also conceivable that stimulation of MLP transcription by the UEFy requires a precise adjustment that is not fullfilled in our present heterologous *in vitro* system. Indeed, we have recently demonstrated that the purified UEFy was able to stimulate *in vitro* transcription of the Ad2 MLP in a yeast crude nuclear extract[17], which strongly suggests that the UEFy has in yeast an analogous function to the UEFh in HeLa. However, detection of the stimulatory activity was possible only when the factor was included in a homologous system.

ACKNOWLEDGEMENTS

We are very grateful to P. Chambon for his interest in this work and for very helpful discussions. We thank A. Kempf and J. M. Chipoulet for excellent technical assistance.

REFERENCES

1 N. C. Jones, P. W. J. Rigby and E. B. Ziff, *Genes Dev.*, 2 (1988) 267–281.
2 B. L. Davison, J. M. Egly, E. R. Mulvihill and P. Chambon, *Nature (London)*, 301 (1983) 680–686.
3 N. Nakajima, M. Horikoshi and R. G. Roeder, *Mol. Cell. Biol.* 8 (1988) 4028–4040.
4 N. G. Miyamoto, V. Moncollin, J. M. Egly and P. Chambon, *EMBO J.*, 4 (1985) 3563–3570.
5 M. Sawadogo and R. G. Roeder, *Cell*, 43 (1985) 165–175.
6 M. Horikoshi, M. F. Carrey, H. Kakidani and R. G. Roeder, *Cell*, 51 (1988) 251–260.
7 B. Cavallini, J. Huet, J. L. Plassat, A. Sentenac, J.-M. Egly and P. Chambon, *Nature (London)*, 334 (1988) 77–80.
8 S. Buratowski, S. Hahn, P. A. Sharp and L. Guarente, *Nature (London)*, 334 (1988) 37–42.
9 J. L. Manley, A. Fire, A. Cano, P. A. Sharp and M. L. Gefter, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 3855–3859.
10 V. Moncollin, N. G. Miyamoto, X. M. Zheng and J. M. Egly, *EMBO J.*, 5 (1986) 2577–2584.
11 J. T. Kadonaga and R. Tjian, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 5889–5893.
12 S. Dézeléé, F. Wyers, A. Sentenac and P. Fromageot, *Eur. J. Biochem.*, 65 (1976) 543–552.
13 M. Fried and D. M. Crothers, *Nucleic Acids Res.*, 9 (1981) 6505–6525.
14 J. M. Egly and E. Boschetti, in I. Chaiken, I. Parikh and M. Wilchek (Editors), *Affinity Chromatography and Biological Recognition*, Academic Press, New York, 1983 pp. 447–453.
15 V. Moncollin, A. Kempf and J. M. Egly, *J. Virol.*, (1990) in press.
16 D. A. Hager and R. R. Burgess, *Anal. Biochem.*, 109 (1980) 76–86.
17 V. Moncollin, R. Stalder, J. M. Verdier, A. Sentenac and J. M. Egly, *Nucleic Acids Res.*, submitted for publication.
18 A. C. Lennard and J. M. Egly, *EMBO J.*, 6 (1987) 3027–3034.