Characterization of a cellular factor which interacts functionally with Oct-1 in the assembly of a multicomponent transcription complex

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ABSTRACT

Induction of transcription of the immediate-early (IE) genes of herpes simplex virus involves the assembly of a DNA-binding complex containing the viral protein Vmw65 and the cellular transcription factor Oct-1. We show that Oct-1 is not sufficient for complex formation and that another cellular factor(s) which is absolutely required for complex formation can be separated from Oct-1 under native conditions. We have purified this factor by approximately 100-fold using DNA-cellulose, ion-exchange and size-exclusion chromatographies. The assay used throughout the purification procedure follows the ability of the cellular factor to form a complex when added to purified Oct-1, Vmw65 and an IE specific DNA probe. The complex forming factor (CFF) had a sedimentation coefficient of about 4.4 S (i.e. molecular mass of about 70K, under non-denaturing conditions) and the polypeptide profile of highly purified CFF demonstrated two major species with molecular masses of 80K and 70K. Unequivocal association of either of these two species with CFF activity could not presently be demonstrated due to the sensitivity of CFF to denaturation. CFF, when tested on its own or in the presence of Vmw65, did not bind to the IE-specific consensus motif. We have also used deletion mutants of Oct-1 to show that the POU domain of this protein was sufficient for CFF-dependent complex formation with Vmw65. Deletions of the POU specific region of Oct-1 significantly reduced the complex forming ability, although detectable levels of complex were reconstituted using Vmw65, CFF and just the homeodomain of Oct-1.

INTRODUCTION

Evidence accumulating from many studies indicates that differential transcriptional regulation of gene expression is accomplished by the selective recruitment of different combinations of factors into multicomponent transcription complexes. While certain factors possess both DNA binding and transcription activation domains other factors may possess only an activation domain and are assembled into DNA binding complexes by protein-protein interactions with factors which recognize specific target sequences (1,2).

The coordinate induction of the immediate-early (IE) genes of herpes simplex virus (HSV) provides one of the best characterized examples of this latter mode of regulation. The virion protein Vmw65 (VP16) induces transcription of the IE genes of HSV dependent on the presence of a specific consensus motif, the TAATGARAT motif, which is present upstream of the IE promoters in one or more copies (3−11). However Vmw65 does not independently bind to this motif and does not exhibit non-specific DNA binding properties (12,13). Instead Vmw65 is recruited into a DNA binding complex (TRF.C) on the TAATGARAT motif by virtue of an interaction with the cellular transcription factor Oct-1 (TRF) which binds the consensus octamer sequence overlapping the TAATGARAT motif (13,14,15,16,17). The selective recruitment of Vmw65 onto the virus IE regulatory sequence is achieved by the presence of additional sequences, the GARAT region, which flanks the octamer binding site in the TAATGARAT motif (18,19). Thus, studies of this system revealed a mechanism to explain the involvement of the same transcription factor in the expression of genes which are not coordinately regulated. Genes which contain the consensus octamer motif but not the flanking GARAT sequence will not be induced by Vmw65, but can be regulated by an octamer binding factor acting via a different mechanism (20).

Significant progress has been made on structural aspects of proteins involved in formation of the complex. The regions of Vmw65 required for assembly of the Oct-1 containing complex are distinct from those required for transcriptional activation of the IE genes (21−24). Several studies have demonstrated that the carboxy terminus of Vmw65 represents an autonomous acidic activation domain which when fused to DNA binding proteins will consequently induce expression of genes containing the appropriate cognate sites (25,26). We have shown directly that this acidic domain is not required for the interaction with Oct-1 and the assembly of the DNA binding complex (22). Refined

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analyses of the Oct-1 protein have indicated that the complete POU domain of this protein is sufficient for complex formation and that residues within the homeodomain are absolutely critical (17).

Nonetheless many questions concerning assembly of the Vmw65-Oct-1 complex remain to be answered. In particular previous results indicate that at least one (19) and possibly two (35) separate cellular components are required in addition to Oct-1 for complex assembly with Vmw65. These additional components have not been characterized however and it is clear that detailed analysis of the multiplicity of interactions that occur during the complex assembly in a reconstituted *in vitro* system requires all participating components to be identified and available in a purified form. In this work we confirm the previous observations on the requirement for an additional component for the Vmw65 transcription complex assembly. We also describe a purification procedure for this factor and characterization of its molecular and functional properties.

MATERIALS AND METHODS

Preparation of Vmw65

Vmw65 was prepared from VERO cells infected with HSV-1 [MP]. For this purpose, 4×10^8 cells were infected at a multiplicity of 2−4 p.f.u per cell. Cells were harvested after 16 hours, suspended in four volumes of buffer A (10 mM Tris-HCl, pH 7.5; 10 mM KCl; 1.5 mM $MgCl_2$; 2 mM EDTA; 0.5 mM PMSF; 5 μg/ml of pepstatin A, aprotinin and leupeptin) and disrupted by sonication (3×15 second pulses). The sonicate was adjusted to 125 mM KCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 25 mM Tris-HCl (pH 7.5), and sedimented by centrifugation for 1 hour at 30,000 g. The resulting supernatant was diluted 5-fold with buffer B (25 mM Tris-HCl 7.5, 50 mM NaCl, 1 mM EDTA, 0.01% NP-40 and 0.5 mM PMSF) and loaded on a Mono Q column (HR 5/5, Pharmacia LKB) equilibrated with the same buffer. The column was washed with 5 ml of buffer B, followed by a step elution with 10 ml of 0.2M NaCl in buffer B. The elution was continued with 25 ml of a linear gradient of 0.2M−1M NaCl at a flow rate of 1 ml/min and 1 ml fractions were collected. The fractions were assayed for the presence of Vmw65 by Western blotting using the mouse monoclonal antibody, LP1 (27). Fractions containing the peak of Vmw65 (eluted with approximately 0.4M NaCl) were combined and stored at −70°C.

Preparation of octamer binding protein (TRF) from HeLa cell nuclear extract: separation from CFF activity

The procedure for preparation of HeLa cell nuclear extract was essentially as described by Dignam et al. (28). Partial purification of TRF was performed by either of the following methods: octamer-specific DNA affinity chromatography, size-exclusion chromatography on Superose 12 or chromatography on a DNA cellulose column. Octamer-specific DNA affinity chromatography was performed according to published procedures (29). A column (1×1.4 cm) was loaded with an aliquot of HeLa cell nuclear extract (about 4−8 mg of protein) washed and eluted with a stepwise gradient of 0−0.8M KCl. Fractions containing TRF free from CFF activity were eluted with 0.3−0.4M KCl. For size exclusion chromatography, 200 μl aliquots of HeLa cell nuclear extract (1−2 mg of protein) were applied to a Superose 12 columnn (HR 10/30, Pharmacia LKB) pre-equilibrated with a buffer containing 20 mM HEPES-KOH, pH 7.9, 0.2 mM EDTA, 0.1M KCl, 0.5 mM DTT, 0.5 mM PMSF and 20% glycerol. The sample was eluted with the same buffer at a flow rate of 0.5 ml per minute and 0.5 ml fractions were collected. Fractions containing the peak of TRF, free from CFF activity, had an apparent molecular mass of about 100,000. Chromatography on DNA-cellulose (Sigma), also used as a purification step (step 1) for CFF activity, is described below. Fractions containing TRF were eluted with 0.35−0.40M NaCl. Aliquots containing partially purified TRF were stored at −70°C.

Purification of CFF from HeLa cell nuclear extract

Large scale preparation of HeLa cell nuclear extracts (from approximately 5×10^9 cells) was according to Dignam et al. (28), except that at the final stage extracts were dialyzed against buffer C (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5 mM PMSF, 0.06% 2-mercaptoethanol and 10% glycerol) and immediately subjected to the following purification procedure:

Step 1. Chromatography on a DNA cellulose column. A DNA cellulose column (2.6×8 cm) pre-equilibrated with buffer C, was loaded with 50−80 mg of nuclear extract protein, washed with 150 ml of buffer C, and then eluted with 150 ml of a linear gradient of 0.05−0.40M NaCl at a flow rate of 0.2 ml per min., and 5 ml fractions collected. The peak fractions, approximately 50 mls, of the CFF (see below for assay) were dialyzed overnight against buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 0.06% 2-mercaptoethanol, and 0.5 mM PMSF (buffer D).

Step 2. Anion-exchange chromatography on a Mono Q column. After dialysis, the appropriate sample from step 1 was loaded onto a Mono Q column, washed with 5 ml of buffer D, and eluted with 20 ml of a linear gradient of 0.05−0.60M NaCl followed by 5 ml of 1M NaCl. The column was eluted at a flow rate of 1 ml per min. and 1 ml fractions were collected. Fractions containing the peak of CFF activity were combined and concentrated to 200−300 μl with Aquaside (Calbiochem).

Step 3. Size-exclusion chromatography on a Superose 12 column. The CFF fraction from step 2 was loaded onto a Superose 12 column pre-equilibrated with buffer containing 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.1M KCl, 0.5 mM DTT, 0.5 mM PMSF and 5% glycerol. Conditions of elution were as described above for preparation of TRF. The peak fractions of the CFF, if not used immediately, were stored at −70°C, under which conditions the activity was stable for several weeks at least.

Glycerol density gradient analysis

The sedimentation coefficient of the CFF was determined on 5−30% (v/v) linear gradient of glycerol in a buffer containing 20 mM HEPES-KOH pH 7.9, 1 mM EDTA, 1 mM DTT and 100 mM KCl. A sample (0.2−0.4 ml aliquot) of CFF obtained after step 2 of the purification procedure described above, was layered over the 3.6 ml gradient and subjected to centrifugation at 4°C for 18 hours at 300,000×g. Aldolase, bovine serum albumin and myoglobin were subjected to concurrent centrifugation on separate gradients. Fractions of about 120 μl were collected and aliquots (5 μl) assayed for CFF activity.

Analysis of CFF and octamer binding (TRF) activity using electrophoretic mobility shift assay

The assay mixture for TRF DNA binding activity contained in a total volume of 40 μl: 25 mM HEPES-KOH pH 7.9, 100 mM

NaCl, 10% glycerol, 0.5% NP-40, 1 mM DTT, 1 mM EDTA, 5.0 mg/ml of bovine serum albumin, and 1−2 μg of poly(dI.dC)-poly(dI.dC). To assay for the fractionation of CFF activity, Vmw65 and TRF were added to the assay mixture and the concentration of NaCl decreased to 50 mM. After 10 minutes incubation at room temperature, end labelled oligonucleotide probe TAAT24 (or, where indicated, TAAT22) was added and incubation continued for a further 20 minutes. Samples were then loaded onto 4% non-denaturing polyacrylamide gels (acrylamide:bisacrylamide, 39:1). Electrophoresis was performed for 2.5−3 hours at 200 V in a buffer containing 0.5×TBE. Electrophoretic mobility shifts (30) of the labelled probe into bands designated as 'TRF.C' (in the assay for CFF) or as 'TRF' (in the assay for TRF) were visualised by autoradiography of the dried gels. For quantitive measurements, the areas of interest were cut out of dried gels and Cerenkov radiation of ^{32}P was measured with an efficiency of approximately 30%. In all cases the probe was present in excess amounts but was frequently run off the bottom of the gel to facilitate separation of TRF.C. Oligonucleotide probes TAAT24; CCATGGAGATCT-CGTGCATGCTAATGATATTCTT, and TAAT22; CTAGA-GATCTCGTGCATGCTAATCCGCGACTTT have been previously described (18,22). Briefly, TAAT24 contains a TAATGARAT element from the HSV IE110K promoter which has a good overlapping consensus octamer sequence (ATGC-TAAT). Oligonucleotide TAAT22 has transversions at the 3′ end of the TAATGARAT element. Both probes were end labelled with α-^{32}P dATP using the Klenow fragment of DNA polymerase 1.

Overexpression and preparation of Oct-1, Oct-2 and Oct-1 mutant proteins

Construction of recombinant vaccinia viruses, preparation of extracts of infected HeLa cell and purification of the POU and homeodomain was previously described by Verrijzer et al. (31).

Other methods

Protein concentration was determined using the BioRad protein assay. SDS polyacrylamide gel electrophoresis was according to the method of Laemmli (32).

RESULTS

1. Separation of TRF from an additional factor required for the formation of a DNA-binding complex.

It has been previously shown (13−17, 19) that formation of a DNA-binding complex containing Vmw65 on HSV IE sequences required the cellular octamer binding factor (Oct-1, TRF). To examine whether this octamer binding factor was sufficient to support formation of this complex (TRF-C), the protein was subjected to different purification procedures and DNA binding activity of the purified fractions assayed in the absence and presence of Vmw65. In Figure 1, the ability of a crude nuclear extract to form TRF.C upon addition of Vmw65 was compared with an equivalent amount of partially purified TRF DNA binding activity obtained by octamer-specific DNA affinity chromatography. While the formation of TRF.C was readily detected in the crude preparations (Figure 1, lanes 1 and 2), formation of the complex was completely undetectable using the preparation of partially purified TRF (Figure 1, lanes 3−4). The same negative result was obtained with TRF purified from the nuclear extract by size-exclusion chromatography and

Fig. 1. Partially purified TRF is not sufficient for TRF.C formation. Reaction mixtures analysed in this DNA-shift assay contained: (1) 1μl of HeLa cell nuclear extract; (2) 1μl of HeLa cell nuclear extract plus 1μl of Vmw65; (3) 5μl of partially purified TRF; (4) 5μl of partially purified TRF plus 1μl of Vmw65. The purification of TRF by sequence-specific affinity chromatography and of Vmw65 by ion exchange chromatography is described in Materials and Methods. Gel retardation analysis performed as described in Materials and Methods except that for the affinity purified TRF the amount of non-specific competitor was reduced to 0.1μg. Positions of migration of TRF and TRF.C are indicated.

chromatography on DNA-cellulose. Similarly, Oct-1 protein overexpressed using recombinant vaccinia virus and purified by ion exchange chromatography was not sufficient for TRF.C formation (not shown). This Oct-1 protein was not only able to bind to the specific oligonucleotide probe but was also functional in an *in vitro* assay for adenovirus replication (31). Together these data suggested that functional properties of TRF were not affected during the purification procedures and indicated that another component from the nuclear extract was required for complex formation and that this component was separated from TRF during the purification.

To test this proposal, all the fractions that were obtained from the DNA cellulose chromatography step and originally assayed for TRF binding activity (Figure 2B) were also analysed for formation of TRF.C in an assay developed specifically for this purpose (Figure 2C). The assay mixture, to which aliquots of the column fractions were added, contained partially purified TRF and Vmw65, in addition to the normal constituents. The results presented in Figure 2 demonstrated that fractions (12−26) eluted separately from the peak of TRF (fractions 30−38) contained a factor which restored TRF.C formation. We designated this factor accordingly as *c*omplex *f*orming *f*actor (CFF).

Similar experiments as those described in Figure 2 were performed with the different fractions resulting from partial purification of TRF by octamer-binding DNA affinity chromatography or by size-exclusion chromatography (see below). These experiments showed that CFF activity was separated from TRF by these methods also.

When different samples were analysed for CFF activity, several control conditions routinely accompanied the assay. Firstly, it was important to exclude the possibility that what had been identified as TRF.C was in fact a DNA-binding protein which fortuitously migrated at the same position as TRF.C in a gel

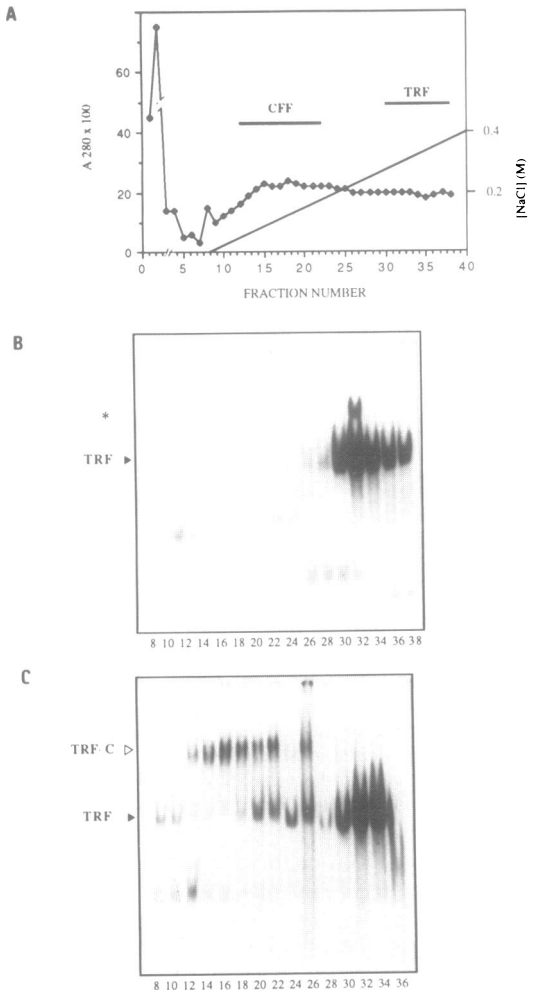

Fig. 2. DNA-cellulose chromatography of HeLa cell nuclear extract. A) HeLa cell nuclear extract containing 65mg of protein was subjected to chromatography on DNA-cellulose (see Methods). A measure of the protein concentration (absorbance at 280 nm) is indicated by solid circles and the NaCl concentration by the solid line. B) Aliquots (1μl) of the fractions indicated were analysed for TRF binding in the reaction mixture described in Methods. The arrow indicates position of TRF migration. Position of a slower migrating band is also marked (*). The latter, here visible in some of the fractions of TRF peak, can be distinguished from TRF.C: it has different mobility (slightly faster) and it is not dependent on the addition of Vmw65. Appearance of this band is dependent on TRF concentration, total protein concentration, and ionic conditions in the reaction mixture. When assayed under slightly different conditions (see C), the band was not detected. C) Another aliquot of the column fractions (5μl) was analysed for TRF.C formation in the presence of 5μl TRF (prepared by size-exclusion chromatography, see Methods) and 1μl of Vmw65. Other components of reaction mixtures are described in Methods. The arrows indicate migration of TRF and TRF.C.

retardation assay. As shown in Figure 2B (fractions 12−26), none of the fractions containing CFF activity showed a band migrating at the same position as TRF.C, when assayed in the absence of Vmw65 and TRF. Other controls included omissions of either Vmw65 or TRF from the reaction mixture and the examination of DNA-sequence specificity for the complex formation; these are described further below when employed to analyse properties of highly purified CFF preparations (section 4).

2. Partial purification of CFF

The purification procedure for CFF combined DNA cellulose chromatography described above (Figure 2) with anion-exchange and size-exclusion chromatographies (see Methods for details). The chromatography on DNA-cellulose was chosen as the first purification step for several reasons. When compared to other methods that separated CFF from TRF, DNA cellulose chromatography achieved the best purification of CFF itself (10-fold). In addition, this method removed DNA-binding proteins that had higher affinities for DNA, thus eliminating their potential interference in the assay for CFF activity.

Fractions containing the peak of CFF activity (Figure 2C, fractions 14−22) were pooled and subjected to chromatography on a Mono Q anion exchange column. CFF activity bound weakly to the column and was eluted between 50 and 100 mM NaCl (not shown). Purification on a Mono Q column was also useful as a method of concentrating CFF activity; the CFF activity peak represented about 1/20 of the sample volume loaded onto the column. Finally size-exclusion chromatography on Superose 12 (see Section 3) resulted in further effective separation of CFF from other proteins (Figure 3).

The relative amounts of CFF in the fractions after the different purification steps were determined in a standard assay with Vmw65 and TRF present in non-limiting concentrations. The assays were performed simultaneously using the same preparation of radiolabelled probe to avoid error due to difference in probe specific activity (Figure 4). Samples (1 μl) of CFF from each of the chromatographic steps are present in all four tracks, lanes 3−6 for the DNA cellulose peak, lanes 7−10 for the Mono Q peak and lanes 11−14 for the Superose 12 peak. Each CFF peak was assayed alone (lanes 3, 7, 11) or together with only TRF (lanes 4, 8, 12) or with TRF plus Vmw65 (lanes 5,6, 9, 10, 13 and 14). Under these conditions the amount of TRF.C formed was proportional to the concentrations of CFF in these assays until at least 500 cpm had been driven into the complex. Estimates of CFF activity in terms of cpm per μl of fraction were then calculated. The concentration of the total protein in the fractions was also determined and the quantitative result of purification steps are summarized in Table 1. The procedure resulted in an overall purification of 95-fold with a yield of 3%. The specific activity of the final CFF preparation was about 10 pmoles of the oligonucleotide probe shifted into TRF.C per mg of protein. Although this value falls within the broad range of specific activities published for the purified transcription factors the direct comparison cannot be applied. The formation of TRF.C, here taken as a measure of CFF activity, involves different and more complicated interactions than direct DNA-binding.

The purity of the fractions containing CFF obtained after different purification steps was analysed by SDS-gel electrophoresis (Fig. 5). The gel shows the protein profile of the starting nuclear-extract (lane 1), DNA cellulose peak (lane 2) Mono Q peak (lane 3) and the peak from the final gel filtration step (lane 4). Clearly the procedure has resulted in a substantial purification of CFF activity with respect to the polypeptide profile. Only two major polypeptides with molecular masses of 80K and 70K were present in the final preparation and the appearance of these species correlated with CFF activity in the fractions during the final purification step (size exclusion chromatography). However 2 minor species with molecular mass of 145K and 30K were also detected in the purified CFF preparation and in order to more definitively correlate CFF activity with one or other of the polypeptides present in the fraction, a method combining extraction of protein from SDS gels with renaturation of its activity was explored (33). Despite exhaustive attempts we were unable to regain CFF activity after dissolution in 6M guanidine-HCl and unable to assign CFF

TABLE 1

	Volume	Protein		Activity*		Specific Activity	Purification	Yield
	ml	mg/ml	total mg	cpm/ml	total cpm	cpm/mg	fold	%
Nuclear extract	10	6.50	65	162,857	1,628,570	25,055	1	100
DNA cellulose	50	0.09	4.50	22,800	1,140,000	253,333	10	70
Mono Q	3	0.135	0.40	78,200	234,500	586,250	23	14
Superose 12	2	0.010	0.02	23,800	47,600	2,380,000	95	3

* CFF activity was expressed as cpm of ^{32}P labelled T24 shifted into TRF.C under standard assay conditions. Quantitation was carried out as described in Fig. 5.

activity to a single polypeptide species. Nonetheless from the protein profile of the purified fractions and the correlation of species with activity during fractionation, we think it likely that the 80K or 70K species (or both) constitute the complex forming factor.

3. Molecular properties of CFF

Size-exclusion chromatography (Figure 3) described above as an effective purification step also allowed an estimation of apparent molecular mass of CFF. A size of between 400,000 and 500,000 was estimated for the peak of CFF activity present in fractions 3 and 4 (Figure 3). An increase of NaCl concentration from 100 to 500 mM in order to minimize non-specific protein-protein interactions did not significantly change this elution profile. The same size was estimated when this method was used to separate CFF from TRF present in the crude nuclear extract. (Under these conditions TRF had an apparent molecular mass of about 100,000).

Sedimentation analyses of CFF preparations were performed by centrifugation in glycerol gradients (5−30%). A sedimentation coefficient of CFF present either in crude nuclear extract (not shown) or partially purified (Figure 6, peak fraction 18) had value of about 4.5 S. This sedimentation coefficient would correspond to molecular mass of about 70,000 for a globular protein with an average partial specific volume and degree of hydration, a value considerably different from the molecular mass obtained by gel filtration. The most likely explanation for the discrepancy in molecular size estimated by two different methods is that a size-exclusion chromatography on Superose 12 could preserve formation of homo- or hetero-multimers that became dissociated during glycerol-gradient sedimentation and there is precedent for this in the analysis of several transcription factor complexes (34). Alternatively, CFF could interact with Superose 12 in a way that affected its elution profile based only on size-exclusion. The latter would result in an incorrect estimation of CFF molecular size.

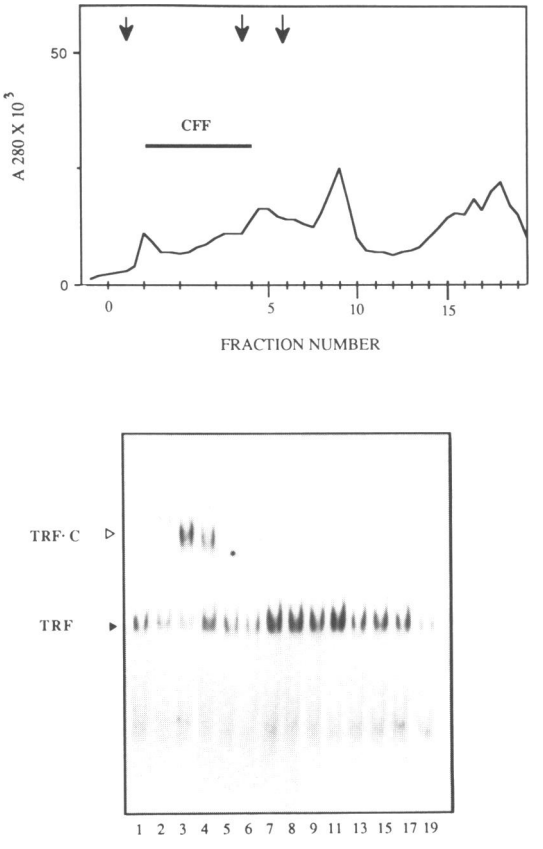

Fig. 3. Size-exclusion chromatography on Superose 12. A preparation of CFF purified by DNA-cellulose and anion-exchange chromatographies was subjected to chromatography on Superose 12. Aliquots (5μl) of the fractions indicated were analysed for TRF.C formation. Arrows, from left to right, indicate positions of elution of blue dextran, ferritin and catalase.

4. Functional properties of partially purified CFF

Our earlier studies (18) demonstrated that assembly of a DNA-binding complex requires not only the core octamer binding site, but also flanking sequences which are dispensable for TRF binding alone. Thus, transversions in the 3′ region of the TAATGARAT motif present in the wild-type oligonucleotide resulted in an oligonucleotide (TAAT 22) to which TRF was still bound but no TRF.C was formed. This oligonucleotide was used to examine sequence-specificity of the complex formed in the presence of partially purified CFF. As shown in Figure 7, addition of CFF activity to the reaction mixture containing the mutant TAAT22 did not support formation of TRF.C (lane 4) while very efficient complex formation was observed when wild-type oligonucleotide TAAT24 was used (lane 2). Note that, as

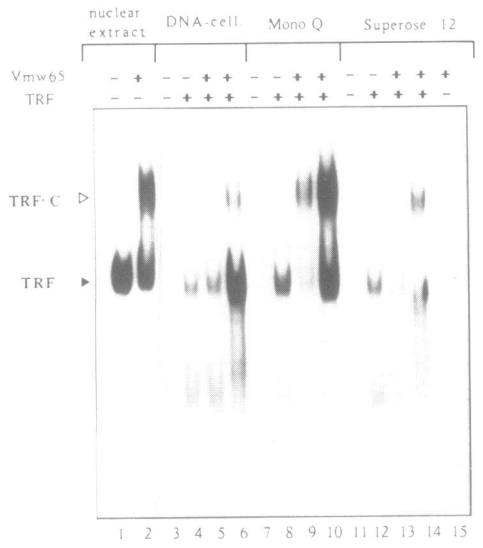

Fig. 4. CFF activity after different purification steps. Samples containing CFF activity from each step of purification described in Table 1, were analysed in an electrophoretic mobility shift assay. HeLa cell nuclear extract (0.3μl aliquots) was analysed in the absence (1) or presence of Vmw65 (2). The samples (1μl aliquots) from DNA-cellulose chromatography (3–6), chromatography on a Mono Q column (7–10) and chromatography on a Superose 12 column (11–14) were assayed without TRF and Vmw65 (3,7 and 11), with TRF only (4,8 and 12) or under the standard conditions with both TRF and Vmw65 (5,6,9,10,13 and 14). In assays 6, 10 and 14 amounts of TRF added were comparable with the amount present in the sample of HeLa cell nuclear extract. The sample obtained after purification on Superose 12 was also analysed in the presence of Vmw65 only (15). Bands corresponding to TRF.C were excised from the gel and quantitated as described in Methods. Values determined for TRF.C were: 210cpm in lane 2; 65cpm in lane 5; 114cpm in lane 6; 186cpm in lane 9; 391cpm in lane 10; 61cpm in lane 13; 119cpm in lane 14. These values represented 70% or less of the maximal amounts of TRF.C that could be generated in the presence of higher concentrations of CFF.

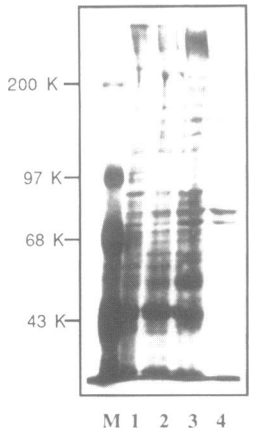

Fig. 5. Protein content after different purification steps. Coomassie Brilliant Blue stained profile of the proteins present in samples from each step of purification of CFF described in Table 1. Samples were analysed on a 10% SDS-polyacrylamide gel. The samples applied were from: (1) HeLa cell nuclear extract; (2) DNA-cellulose chromatography; (3) chromatography on a Mono Q column; (4) chromatography on a Superose 12 column. The position of molecular weight standards is indicated (M).

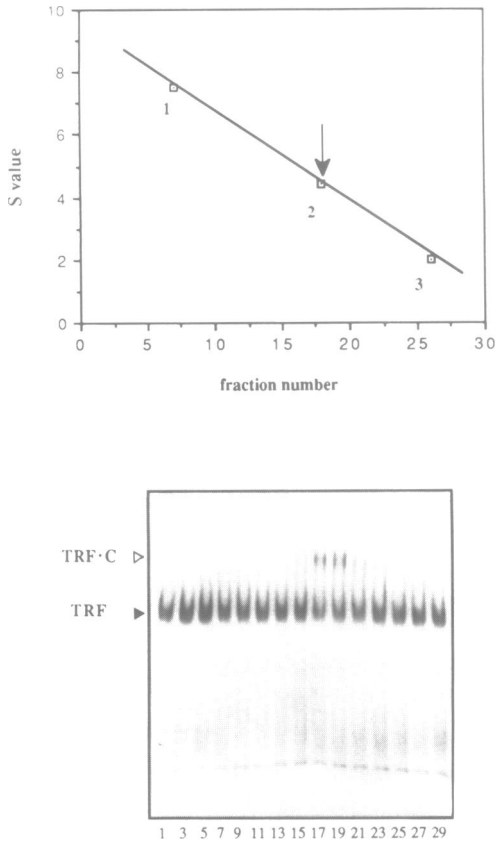

Fig. 6. Sedimentation analysis of CFF activity. A preparation of CFF purified by chromatography on a DNA-cellulose and Mono-Q column was subjected to centrifugation on 10–30% linear glycerol gradient. Aliquots (5μl) of the gradient fractions were assayed for TRF.C formation (bottom panel). The position of sedimentation of the protein standards a, aldolase (7.35S); 2, bovine serum albumin (4.2S) and 3, myoglobin (2.0S) is presented graphically. The solid arrow indicates the peak of CFF activity.

previously demonstrated, mutation of the GARAT region did not affect independent TRF binding (compare lanes 1 and 3). These results demonstrated that the complex reconstituted in the presence of CFF and that in HSV infected cells have the same requirements for the flanking GARAT region in the oligonucleotide probe.

Considering that the formation of TRF.C had this additional DNA sequence requirement compared to TRF alone, it was possible that CFF or CFF/Vmw65 complex was bound to the 3′ flanking GARAT sequences. In order to test this, CFF preparations after different purification steps were analysed for binding of the wild-type oligonucleotide probe in the absence of TRF and Vmw65. As illustrated in Figure 4 (lanes 3, 7 and 11), no band-shift that correlated with the CFF activity was observed. Furthermore CFF preparations did not influence the binding properties of TRF in the absence of Vmw65 (Figure 4, lanes 4, 8 and 12). Addition of Vmw65 (which on its own does not bind to DNA) to the final preparation of CFF did not result in appearance of an oligonucleotide binding activity (Figure 4, lane 15). Thus, under these different conditions CFF did not bind independently to the GARAT region. The binding of TRF was not influenced by the presence of either CFF or Vmw65 alone and formation of TRF.C required the presence of all components (TRF, CFF and Vmw65). Although CFF may contact the GARAT region in the complete complex the only functional assay presently available for CFF is its ability to promote the formation of a DNA binding complex dependent on TRF and Vmw65.

The data presented in section 2 (Figure 2) showed that CFF

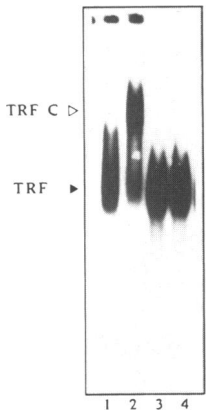

Fig. 7. Nucleotide-sequence requirements for complex formation dependent on CFF. TRF binding and TRF.C formation was analysed by an electrophoretic mobility shift assay using wild-type oligonucleotide TAAT 24 (lanes 1 and 2) or oligonucleotide with transversions in 3′ region of TAATGARAT element TAAT 22 (lanes 3 and 4). The reaction conditions were as in the standard assay for CFF-dependent complex formation using semi-purified TRF and Vmw65. CFF (5ml aliquot of a sample from the final purification step) was added to reaction mixtures analysed in lanes 2 and 4.

present in the crude nuclear extract bound weakly to DNA-cellulose. Partially purified CFF, separated from TRF, possessed the same binding ability for this non-specific ds DNA (not shown). This suggested that CFF could bind the DNA directly, without interacting with another DNA-binding protein such as TRF. However, CFF also bound weakly to some cation-exchange columns (e.g. Mono S, FPLC) and thus, the interaction with DNA could be of a similar nature (i.e. interaction with negative charges) rather than based on an affinity for DNA.

5. Assembly of the DNA binding complex using mutant Oct-1 proteins

Previous results from Stern et al. (17) attempted to identify requirements within the Oct-1 protein for complex formation with Vmw65. This work indicated that the complete POU domain was sufficient, and that residues within the homeodomain of Oct-1 were crucial for complex formation. However complex formation was assayed in a transcription-translation system and was comparatively inefficient presumably due to the absence of CFF. It was possible that residues outside the POU domain were required for efficient CFF-dependent complex formation. To analyse this, full length or mutated versions of Oct-1 were expressed in a recombinant vaccinia virus system purified from the cytoplasm of infected cells and compared with TRF, affinity purified from uninfected nuclear extracts, for the ability to promote complex formation in the presence of Vmw65 and CFF.

The mutant proteins (Figure 8A) had deletions at the amino-terminal end (proteins encompassing residues 212−743 and 1−23/269−743), the carboxy-terminal end (protein encompassing residues 1−440) or both (POU domain, residues 1−23/269−440); another deletion mutant contained only one POU subdomain, the homeodomain (residues 369−440). Our results (Figure 8) demonstrate that each of the mutant proteins containing deletions in either carboxy- or the amino-terminal region up to the POU domain, retained the ability to bind to the oligonucleotide probe (Figure 8B, lanes 1−4) and to form a multicomponent DNA-binding complex upon the presence of CFF (Figure 8B, lanes 6−9). Furthermore in quantitative dose response experiments using highly purified preparations of TRF

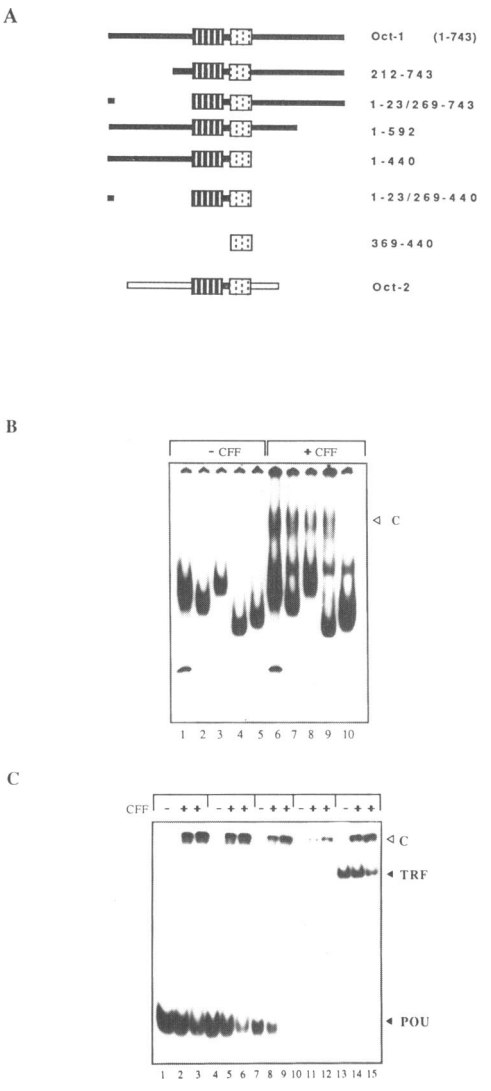

Fig. 8. Deletion mapping of Oct-1 domain required for the complex formation. A) Schematic illustration of Oct-1, Oct-1 mutations and Oct-2 protein. Boxed regions indicate the location of the POU-specific region (vertical bars) and that of homeodomain (dots) as determined by Sturm et al. (36). B) Preparations (2μl of infected cell cytosol) of Oct-1 deletion mutants (lanes 1−4) and Oct-2 protein (lane 5) were analysed for DNA-binding and complex formation in the presence of Vmw65 only (left hand panel) or both, Vmw65 and CFF (right hand panel). CFF was purified by size-exclusion chromatography (5μl aliquot was used). Note that a small amount of contaminating TRF was present in the CFF fraction but this did not influence the assay since no TRF.C was formed when Oct-2 was tested with CFF (lane 10). C) Oct-1 mutant protein containing POU domain, expressed in recombinant vaccinia virus system and further purified, was compared with TRF for the ability to form complex. In this case, 8% polyacrylamide gel (acrylamide: bisacrylamide, 39:1) was used and electrophoresis carried out for 7 hours. A TRF preparation (5μl, prepared by octamer specific DNA affinity chromatography, lanes 13−15) or different amounts of POU domain (10,5,2, and 0.5ng of purified protein, lanes 1−12) were assayed in the presence of Vmw65 only (1,4,7,10 and 13) or both, Vmw65 and CFF (2,3,5,6,8,9,11,12,14 and 15). Two different amounts of CFF, prepared by size-exclusion chromatography, were used: 5μl (2,5,8,11 and 14) and 10μl (3,6,9,12 and 15). Arrows indicate migration of TRF, POU domain, and that of the complexes they form (both, TRF.C and POU.C indicated as C). Using similar amounts of TRF (982 cpm, lane 13) and POU domain (888 cpm, lane 7) DNA binding activities, similar amounts of TRF.C (951 cpm, lane 15) or POU.C (808 cpm, lane 9) were formed upon addition of an equal amount of CFF. This comparison, together with further quantitation of the relative increase in complex formation using two doses of CFF at different input doses of the POU domain (Figure 8C, lanes 1−6) indicates that the POU domain contained all the determinants for efficient recruitment of CFF and Vmw65 into the multimeric DNA binding complex.

and the POU domain (Figure 8c) our results demonstrate that while Vmw65 had no effect on the complete TRF or on the isolated POU domain in the absence of CFF (lanes 13, and 1) the specific activity of the POU domain for complex formation in the presence of CFF was comparable to that of TRF (see Figure 8 legend). These results are in good agreement with those of Kristie et al. (35) who showed that the POU domain was capable of promoting complex formation with Vmw65 dependent upon the addition of crude insect or HeLa cell extracts containing the factors they termed C1 and C2.

Further deletion within the POU domain resulted in a protein that consisted only of the POU homeodomain (residues 369−440). In order to achieve the same binding to the oligonucleotide probe about 100-fold larger amounts of purified homeodomain protein were required. Comparison of equal oligonucleotide binding activites of homeodomain and POU domain in the assay for complex formation (Figure 9) showed that under conditions when 80−90% of POU domain was in the complex (lane 4), only 5−10% of homeodomain was shifted (lane 2). When this difference in complex forming ability was expressed per ng of pure protein, the data showed that 1000−2000 fold larger amounts of the homeodomain were needed for the formation of the same amount of the complex.

As indicated above, formation of the complex by all mutant proteins required the presence of CFF in addition to Vmw65 (Figure 8B, 8C and 9). In contrast to the significant differences in the migration of different Oct-1 mutants, only small differences in the migration of the complexes containing those mutants were detected. This is consistent with the interpretation that the complete complex may have a native size in excess of 500,000 (i.e. CFF alone 300−400,000) and thus have a low mobility which is less sensitive to changes in the mass of one of the components.

Another member of octamer binding family, Oct-2, was tested in the same assay. Unlike Oct-1, no detectable complex was formed in the presence of CFF and Vmw65 (Figure 8B, lane 10).

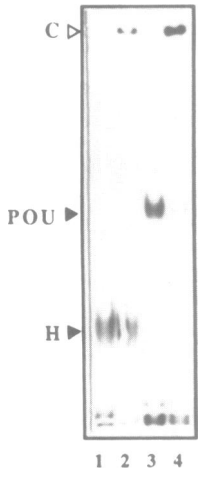

Fig. 9. Complex forming ability of Oct-1 homeodomain and Oct-1 POU domain. Purified homeodomain (100 ng) (lanes 1 and 2) and purified POU domain (1 ng) (lanes 3 and 4) were added into reaction mixtures containing Vmw65 (lanes 1 and 3) or both, Vmw65 and CFF (lanes 2 and 4). The samples were loaded onto 15% polyacrylamide gels (acrylamide:bisacrylamide, 39:1) and electrophoresis carried out at 100V for 16 hours.

DISCUSSION

In this work we describe properties of a cellular factor which interacts functionally with the octamer binding protein (Oct-1): it is required for the assembly of a multicomponent DNA-binding complex on sequences specific for IE promoters of herpes simplex virus and has been designated accordingly as complex forming factor, CFF.

CFF can be readily separated from octamer binding protein not only on the basis of its inability to bind to octamer-specific sequences but also by virtue of its lower affinity for DNA-cellulose and different elution profile on a size-exclusion column. These crude preparations of CFF, when combined with Vmw65 and octamer binding protein, consistently showed the appearance of a single multicomponent DNA-binding complex (designated here as TRF.C). The position of migration of this complex on non-denaturing polyacrylamide gels was identical to the migration of a complex formed in crude nuclear extracts supplemented by Vmw65 (Figure 4) or in HSV infected cells.

A factor (or factors) that have the functional properties of CFF have been recently identified in HeLa cell nuclear extracts depleted of octamer binding proteins (35). Although the physical properties were not examined, Kristie et al. presented evidence for the existence of two independent factors which promoted the formation of two complexes, C1 and C2, in conjunction with Oct-1 and Vmw65. The presence of two factors was inferred from the observations 1, that crude cytoplasmic preparations of Vmw65 expressing insect cells could form a complex with semipurified Oct-1 while purified Vmw65 could not and 2, that HeLa nuclear extracts depleted of Oct-1 could promote the formation of a second complex in extracts containing Oct-1 and crude cytoplasmic Vmw65 from insect cells. The cytoplasmic activity from the insect cells promoting C1 formation was also observed in HeLa nuclear extracts which thus had both C1 and C2 forming activity while the cytoplasmic (or nuclear extracts) from insect cells contained only C1 activity. The DNA binding contacts made in the C1 and C2 complexes were identical and it was proposed that the C1 complex may be a precursor of the C2 complex.

In our hands CFF purifies as a single activity and we have never observed any additional components other than CFF which promotes bona fides Vmw65 dependent complex formation with Oct-1. It may therefore be that CFF is analagous to the C1 activity identified by Kristie et al. and it is possible that in the presence of CFF, Oct-1 and Vmw65 we may detect a second activity requiring the preformed CFF-Oct-1-Vmw65 complex.

In order to study the molecular and functional properties of CFF, the activity was purified about 100-fold from HeLa cell nuclear extract. This rapid procedure combined chromatographies on (i) DNA cellulose, (ii) anion exchange and (iii) size exclusion columns, and is summarized in Table 1. Although purification to homogeneity has not been achieved, CFF preparations obtained using this method were free from octamer binding proteins, free from DNA binding proteins with a higher affinity for DNA and had a relatively high specific activity. Thus, possible interactions of contaminating proteins with CFF activity or the specific oligonucleotide probe have been minimized.

The properties of the DNA binding complex formed in the presence of highly purified CFF, octamer binding protein and Vmw65 were consistent with those previously described for the complex formed by extracts of HSV-infected cells or in a partially reconstituted system. For example, formation of the complex in crude extracts of HSV-infected cells required sequences (G-

ATATT) flanking the core octamer element in the IE110K regulatory motif, thus distinguishing binding specificity of the complex from that of the octamer binding protein. The additional 3′ flanking sequences were also required for reconstitution of the complex using the highly purified components described here (Figure 7). Availability of purified CFF allowed studies of possible interactions between this factor and a wild-type oligonucleotide probe without interference of non-specific DNA binding proteins present in crude preparations. These studies were carried out in the absence or presence of other components of the complex. The data presented here show that CFF does not bind to the 3′ flanking or other sequences present in the oligonucleotide probe in the absence of other protein components (Figure 4). Moreover the combination of CFF and Vmw65 (which is also lacking oligonucleotide binding activity) did not generate sequence specific binding (Figure 4). Thus, if interaction of CFF and/or Vmw65 with 3′ flanking sequences was taking place in the complex, it would have been preceded by protein-protein interaction with the octamer binding protein. Alternatively, this protein-protein interaction could alter (i.e. extend) the binding contacts of the Oct-1 so that the requirement for the GARAT region was due to contacts with Oct-1 only when Oct-1 is in the complex. Direct evidence that the 3′ GARAT region is bound by protein in the multicomponent DNA binding complex has come from the DNAse 1 and orthophenanthroline-Cu cleavage analyses of Kristie et al.(35).

The CFF activity required for the complex formation in the presence of HeLa cell octamer binding protein (TRF), was also required when a structurally defined octamer binding protein, Oct-1 gene product, was used for the assembly of this complex. Similar observations have been reported by Kristie and colleagues using crude CFF-like activity. The relationship between the structure and functions of Oct-1 protein have been studied extensively, including its structural requirements for the complex formation of HSV IE sequences (17). The experimental approach employed replacements of Oct-1 regions with corresponding regions of Oct-2, since the latter was found to be non-functional in assembly of a multicomponent DNA-binding complex. This approach demonstrated that the ability to form complex required the presence of Oct-1 homeodomain in Oct-1/Oct-2 constructs. Consequently, residues that are different in Oct-1 and Oct-2 homeodomains (7 out of 60) have been considered as critical for the complex formation. The purified Oct-1 homeodomain (containing residues 369–440) tested in this work was rather inefficient in the formation of such a complex. This was clearly partly due to the reduced affinity of the homeodomain for the oligonucleotide probe. However, in the presence of high concentrations of the protein significant binding was achieved and it was possible to demonstrate that under these conditions the homeodomain still could not efficiently participate in the complex formation (Figure 9). Consistent with the recent data of Kristie et al. (35) the quantitative analysis of Oct-1 mutant proteins described here (Figure 8B) showed that the intact POU domain (POU homeodomain + POU-specific domain) was as efficient in CFF-dependent complex formation as was the full-length protein. Thus, as indicated previously (17) the sequences within the homeodomain previously recognized as critical does not seem to be sufficient for the complex formation and other residues contained within the POU-specific domain may also participate in interactions required for the complex formation. Such residues are likely to be conserved between Oct-1, Oct-2 and another related protein Pit-1, since their POU-specific domains can be interchanged with little (Pit-1) or no (Oct-2) effect on complex forming ability (17).

Formation of the DNA-binding complex by the complete POU domain, like that of the full-length octamer binding protein, required CFF activity in addition to Vmw65 (Figure 8B). A direct interaction between the POU domain, CFF and/or Vmw65 in solution and within the complex, has yet to be tested and the precise mechanism for the assembly of the multicomponent DNA binding complex on HSV IE promoters has yet to be clarified. The separation of CFF from Oct-1 and its isolation as a highly enriched fraction should now facilitate studies of binding intermediates and order of assembly analyses.

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