Purification of the Cellular C1 Factor Required for the Stable Recognition of the Oct-1 Homeodomain by the Herpes Simplex Virus α-Trans-induction Factor (VP16)*

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The assembly of specific multiprotein complexes on the herpes simplex virus α/IE (immediate early) enhancer elements requires the interactions of the Oct-1 POU homeodomain, the viral αTIF (α-trans-induction factor) (VP16), and at least one additional cellular factor, the C1 factor. The C1 factor interacts directly with αTIF, likely forming an intermediate protein complex that recognizes the Oct-1 homeodomain-DNA complex. The biochemical purification of the mammalian C1 factor suggests that it is composed of multiple subunits of related, but heterogeneous, polypeptides. The interaction of a subset of these polypeptides with αTIF is stimulated by post-translational modifications of the C1 proteins, suggesting that this factor may be a critical target for the regulation of the herpes simplex virus α/IE transcription.

As a class of DNA-binding factors, the homeodomain proteins represent significant regulatory determinants in eukaryotic development. These proteins are involved in the selective transcriptional activation of genes that determine cell-specific fate and promote cell type differentiation (1–4). However, the mechanisms by which distinct homeodomain proteins enact their selective biological functions remain unclear as members of this family contain conserved structures that bind to highly related DNA sequence elements (core motif: TAATNN (5–10)). Recently, analyses of the biology of homeodomain proteins have suggested that determinants within the homeodomain structure specify interactions with other regulatory factors, thus further defining the activity and DNA target specificity (11–16).

The Oct-1 and Oct-2 proteins represent model examples of homeodomain proteins with multiple, distinct functional activities. These proteins are members of a subgroup of the homeodomain family (POU domain family) that contains a highly conserved motif (POU domain) consisting of the POU-specific subdomain and POU homeo-subdomain (17–20). These two independent DNA-binding subdomains are collectively responsible for the high affinity recognition of the octamer DNA element (ATGCATAAT (21–23)). Both Oct-1 and Oct-2 recognize this element with an equivalent DNA binding specificity and affinity. However, they have been implicated in the control of such diverse events as the transcriptional regulation of snRNA genes (24–31), the cell cycle regulation of the histone H1 and H2B genes (32–34), the expression of lymphoid-specific genes (35–43), and DNA replication (44–46). Thus, regulation by the octamer proteins exemplifies two questions of functional specificity in homeodomain biology: the discrimination between two proteins with highly related DNA-binding domains and the mechanism by which a given homeodomain protein can function in a number of distinctly regulated events.

The regulation of herpes simplex virus (HSV) α/IE gene expression by Oct-1, in conjunction with the potent viral transactivating protein αTIF (VP16, ICP25, VMW65), provides a biochemically accessible system for the study of the distinct role of homeodomain proteins. The lytic cycle of herpes simplex virus has been extensively investigated and is composed of a highly regulated cascade of gene expression (Ref. 47, reviewed in Ref. 48). The α/IE (immediate early) genes are expressed upon infection, and their expression is regulated by an HSV-encoded transactivating protein αTIF, α-trans-induction factor (49–51)). This protein is packaged in the tegument structure of the virus, released into the cytoplasm upon infection, and transported to the nucleus where it induces the transcription of the five α/IE genes (52, 53).

Transcriptional regulation by αTIF is dependent upon a reiterated enhancer element (α/IE response element) that is present in the region 5' to the α core promoters (54–59). These elements are recognized by the octamer-binding proteins and are targets for the assembly, in vitro, of multiprotein complexes (C1 and C2) that contain Oct-1, αTIF, and additional cellular factors (C1 and C2 factors (22, 60–67)). In contrast, the highly related Oct-2 protein is not efficiently recognized by the αTIF-C1/C2 factors, resulting in its 10-fold lower potential for the assembly of the C1 complex (65, 66, 68, 69).

As depicted in Fig. 1, the HSV α/IE element is bipartite, consisting of a divergent octamer sequence (ATGCTAAT) and 3'-flanking sequences that are highly conserved among the α/IE elements (55). In the C1 complex, the Oct-1 POU-specific domain recognizes ATGC and the POU homeodomain recognizes TAATGA (22, 23). The second DNA-binding protein, the viral αTIF, recognizes the conserved sequences that were thereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: HSV, herpes simplex virus; DTT, dithiothreitol; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PC, phosphocellulose chromatography.
extend 3′ to the octamer site (22, 68). However, unlike Oct-1, the independent DNA binding affinity of αTIF is very low. Although the protein can cooperatively bind DNA via specific interactions with the Oct-1 homeodomain, this complex is not stable to gel electrophoresis (22, 68, 69).

The formation of a stable C1 complex requires Oct-1, αTIF, and an additional cellular factor (the C1 factor; Fig. 1). The C1 factor does not appear to independently bind the α/IE element (22, 70), but rather, interacts with αTIF in the absence of the other components of the C1 complex (22, 71). Therefore, it is likely that the C1 factor interacts with αTIF to form a stable protein complex that subsequently recognizes the DNA-bound Oct-1 homeodomain. To analyze the nature of the C1 factor, we have biochemically purified this factor from mammalian cell extracts.

**MATERIALS AND METHODS**

**Purification of the Mammalian C1 Factor**—The C1 factor was purified from nuclear extracts prepared from a 347-g frozen pellet of HeLa cells (a gift of R. Wobbe and S. Ludmerer of Merck Sharp and Dohme Corp.). Nuclear extracts were prepared as described (72), except that the extracted nuclei were pelleted at 20,000 X g for 30 min and subsequently reextracted with nuclear extraction buffer + 0.1% Nonidet P-40 for 30 min. The pooled extracts were clarified by centrifugation at 40,000 X g for 1 h in an SW 40 rotor prior to dialysis against buffer A (20 mM Hepes [pH 7.9], 0.5 mM EDTA, 20% [v/v] glycerol, 2.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride) + 100 mM KCl. The protein concentration of the resulting nuclear extract was 8–12 mg/ml.

A 500-ml phosphocellulose (Whatman P11) column (5 × 25 cm) was prepared according to the manufacturer's instructions. The column was washed sequentially with 1.5 liters of buffer B (20 mM Hepes [pH 7.9], 0.5 mM EDTA, 20% [v/v] glycerol, 2.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 3 m urea) + 100 mM KCl, 1 liter of buffer B + 100 mM KCl, and 0.2 mg/ml bovine serum albumin (BSA; Boehringer Mannheim), 1 liter of buffer B + 100 mM KC1, and 5 liters of buffer B + 50 mM KCl. HeLa cell nuclear extract was diluted 4:1 with buffer A + 6 m urea and incubated on ice for 30 min. The extract (1035 ml, 3620 mg of total protein) was loaded onto the phosphocellulose column at 250 ml/h. The column was washed with buffer B + 50 mM KC1 at 500 ml/h, and the C1 factor was eluted with a 2 liters of linear gradient of 50–100 mM KCl at 250 ml/h.

The eluate was monitored until it reached a conductivity equivalent to 85 mM KCl, at which time the column was step eluted with 1 liter of buffer B + 100 mM KCl and 1.5 liters of buffer B + 750 mM KCl. Preparation II consisted of four consecutive phosphocellulose column runs (PC-D, PC-E, PC-F, and PC-H).

A Q Sepharose FF column (5 × 7.5 cm) was prepared by washing sequentially in 0.45 liter of buffer C (20 mM Hepes [pH 7.9], 0.2 mM EDTA, 10% [v/v] glycerol, 3 m urea, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride) + 50 mM KCl, 0.45 liter of buffer C + 100 mM KCl and 0.2 mg/ml BSA, 0.4 liter of buffer C + 100 mM KCl, and 1.5 liter of buffer C + 50 mM KCl. The phosphocellulose column fractions containing the peak of the C1 factor activity were diluted to buffer C + 50 mM KCl (final of 160 mg in 1950 ml) and loaded onto the Q Sepharose FF column at 250 ml/h. The column was washed with buffer C + 50 mM KCl until the A280 nm returned to the base line. The C1 factor activity was eluted with a 0.75-liter linear gradient of 50–250 mM KCl at 200 ml/h, followed by 0.3 liter of buffer C + 250 mM KCl and 0.3 liter of buffer C + 750 mM KCl. Preparation II consisted of two consecutive Q Sepharose FF column runs (QFF-A and QFF-B).

A second Q Sepharose FF column (5 × 5 cm) was prepared as described above. The fractions containing the peak of the C1 factor activity were pooled, diluted to buffer C + 50 mM KCl (final of 24.4 mg in 1300 ml) and loaded onto this column at 200 ml/h. The activity was eluted by washing the column successively with 0.3 liter of buffer C + 200 mM KCl and 0.3 liter of buffer C + 500 mM KCl at 100 ml/h.

The fractions containing the peak of the C1 factor activity were diluted to buffer C + 30 mM KCl (final 19.8 mg in 210 ml) and applied to a Mono Q HR 10/10 column (Pharmacia LKB Biotechnology Inc.), equilibrated in the same buffer, at 0.5 ml/min.

The column was washed with 40 ml of buffer C + 30 mM KCl and was developed with a 56-ml linear gradient of 30–125 mM KCl in buffer C followed by 16 ml of buffer C + 125 mM KCl. The column was subsequently washed with 32 ml of buffer C + 250 mM KCl and 24 ml of buffer C + 750 mM KCl. The C1 factor activity eluted from the Mono Q column in two defined peaks (pool I and pool II).

Pool II was separated into buffer C + 40 mM KCl (2 mg in 32 ml, MSD column; and 1 mg in 10 ml, MLE column, respectively) and applied to a Mono S HR 5/5 column (Pharmacia LKB Biotechnology Inc.), equilibrated in buffer C + 40 mM KCl, at 0.2 ml/min. The columns were washed with 5 ml of buffer C + 40 mM KCl and were developed with an 8-ml linear gradient of 40–200 mM KCl at 0.1 ml/min.

The columns were subsequently washed with 3 ml of buffer C + 200 mM KCl and 3 ml of buffer C + 750 mM KCl.

In all cases, the KCl concentrations of alternate chromatographic fractions were determined by plotting the measured conductivity against the KCl concentration. All values of KCl in the appropriate chromatographic buffer. The protein concentration of each chromatographic fraction was determined by Bio-Rad protein assays, using γ-globulin as a protein standard. The elution of the C1 factor, the determination of the specific activities of the chromatographic fractions were done as described below.

**Electrophoretic Mobility Gel Shift Assays**—The production and purification of the Protein A-Oct-1 POU domain was as described (22). The expression, production, and purification of the wild type αTIF protein from AcNPV-αTIF infected SF9 cells was as described (22, 65) (gift of S. Ludmerer of Merck Sharp and Dohme). Oct-1 (2.5′/3′) DNA probe is equivalent to phage Oct-1 (22). Unless otherwise stated, the reactions for the formation of the C1 complex were done as described (22) and included 0.8 ng (12 fmol) of HSVαO DNA probe, 200 ng of poly[dI-dC]-poly[dI-dC], 20 mM Hepes (pH 7.9), 0.5 mM EDTA, 50–100 mM KCl, 2 mM DTT, 4% Ficoll 400, 500–5000 mIU BSA, 30 ng of purified αTIF protein, 0.3 μl bacterial lysate containing 10–20 ng of PA-Oct-1 POU protein, and the appropriate C1 factor chromatographic fraction in a total of 10 μl.

The reactions were incubated at 30 °C for 20 min and were resolved in 4% non-denaturing polyacrylamide gels (acrylamide: bisacrylamide, 29:1) using 0.5 × Tris/glycine buffer (73, 74). The gels were dried and exposed to Kodak X-Omat film or directly quantified by using a Molecular Dynamics phosphorImager with ImageQuant 3.0/3.1 software.

The C1 factor activity units were calculated by dividing the chromatographic fractions into a C1 complex formation assay such that 50% of the Oct-1-DNA complex was assembled into a C1 complex. The results of all of the column runs were standardized by the direct comparison and quantitation of the activity of each column pool fraction. A standardization factor was calculated based upon the activity of the first phosphocellulose column run. The quantity, in picomoles, of purified C1 factor was calculated by determining the amount of C1 factor that was required to shift 12 fmol of Oct-1-DNA complex into a C1 complex (1 saturation unit is a minimum of 12 fmol of C1 factor).

For the dephosphorylation of the C1 factor, 0.5 μl of HeLa cell nuclear extract (5–5 μg of protein) or 40 saturation units of the purified C1 factor were incubated with 0.2 unit of potato acid phosphatase (Sigma) for 15 min at 25 °C. The reactions were then diluted into a C1 complex formation assay or were prepared for SDS-PAGE.
Gel Filtration Chromatography of C1 Factor—The production of a Protein A-αTIF protein affinity matrix and its use in the affinity selection of the HeLa cell C1 factor was as described (22). For the determination of the molecular mass of the C1 factor, a Superose 12 gel filtration column was equilibrated in buffer D (20 mM Hepes (pH 7.9), 0.5 mM EDTA, 2.5 mM DTT, 5-10% (v/v) glycerol, 200-500 mM KC1, and/or 1% BSA) at 4 °C. The affinity chromatography was diluted to buffer D (100-200 μl) and fractionated in an identical manner. The molecular mass of the C1 factor was then calculated on the basis of its retention volume in comparison to the linear plot of the retention volume versus log molecular weight of the protein standards. The recovery of the C1 factor activity from several column fractionations was 60-80% of the loaded activity.

SDS-PAGE—Extracts and chromatographic fractions, representing 20-50 saturation units of C1 activity, were resolved in denaturing SDS-polyacrylamide gels (16 cm x 0.75 mm, 7-12.5% acrylamide:bisacrylamide, 30:0.6) at 15 mA. Where appropriate, the gels were submerged in silver nitrate. After silver staining, bands corresponding to the C1 factor were transferred to nitrocellulose in a Bio-Rad transblot unit for 1.5 h at 125 V. The resulting dialysates were tested for their ability to support the formation of a C1 complex based upon an anticipated 2-5% renaturation efficiency.

αTIF Protein Blots—The αTIF probe protein was prepared by phosphorylation of 75 μg of purified αTIF protein with 100 units of protein kinase A (catalytic subunit, Sigma) and 500 μCi [γ-32P]ATP (6000 Ci mm⁻¹) in 0.5 ml of kinase buffer (20 mM Tris 7.5, 100 mM NaCl, 12 mM MgCl₂, 2 mM DTT) for 30 min at 37 °C. The labeled protein was dialyzed against 4 × 0.5 liter of 20 mM Hepes (pH 7.9), 0.5 mM EDTA, 10% (v/v) glycerol, 5 mM DTT, 100 mM KC1 for 6-12 h. Alternatively, the polypeptides in the purified C1 factor preparations were resolved in a 9.5% SDS denaturing gel, eluted from the gel, precipitated with acetone, and renatured as described above (75). The resulting dialysates were tested for their ability to support the formation of a C1 complex based on an anticipated 2-5% renaturation efficiency.

RESULTS

Purification of the Mammalian C1 Factor—Several preliminary observations influenced the development of the purification scheme for the mammalian C1 factor. The fractionation of protein extracts by phosphocellulose, DEAE-Sepharose, and S Sepharose chromatography revealed that the C1 activity did not fractionate homogeneously, but uniformly eluted from these columns within a 50-800 mM KC1 gradient (data not shown). This chromatographic behavior suggested that the C1 factor was heterogeneous or was associated with a variety of components in the nuclear extracts.

In several purifications, chromatographic buffers containing urea were used to dissociate protein complexes, thus allowing for the purification of the individual polypeptides (80-82). To test the hypothesis that the C1 factor might fractionate more homogeneously under similar conditions, aliquots of HeLa cell nuclear extract were incubated in the presence of 1-4 M urea at 4 °C for 30 min. These extracts were subsequently dialyzed to remove the urea or directly diluted into a C1 complex formation assay. In the presence of 1 or 2 M urea, the activity of the C1 factor was unaffected, as compared with untreated controls. In contrast, the activity was increased 100% in the presence of 3 M urea and was decreased 30% in the presence of 4 M urea (data not shown). Furthermore, upon fractionation of HeLa nuclear extract by phosphocellulose chromatography in the presence of 3 M urea, the C1 activity eluted from the column within a narrow KC1 concentration range (data not shown, refer to Figs. 2 and 3). The partially purified C1 activity was stable in the presence of 3 M urea for extended periods of time at both 4 and -80 °C. However, removal of the urea from these fractions by dialysis or buffer exchange chromatography resulted in significant loss of the C1 activity and a reversion to the heterogeneous fractionation patterns (data not shown). Therefore, the entire purification of the C1 factor was done in the presence of 3 M urea.

Purification of the mammalian C1 factor was accomplished according to the scheme depicted in Fig. 2 and as briefly described below. HeLa cell nuclear extracts, in the presence of 3 M urea, were applied to a column of phosphocellulose. As assayed by the ability to assemble Oct-1 and αTIF into a stable C1 complex on the HSVeα αTIE response element, the peak of the C1 activity eluted at 90 mM KC1 (Figs. 2 and 3, Table I). This represented a 14-fold purification of 90% of the total activity (Table I). These fractions were applied to a column of Q Sepharose FF (Q Sepharose FF-1), and the C1 activity was eluted from the column at 100-110 mM KC1 (Fig. 2), resulting in a 14-fold purification of 108% of the chromatographed activity (Table I). The partially purified C1 activity was rechromatographed in a second column of Q Sepharose FF (Q Sepharose FF-2; Fig. 2). Elution of the C1 factor in a single 200 mM KC1 step resulted in a 1.7-fold purification of 171% of the applied activity (Table I). The significant increase in the C1 activity during this fractionation stage probably reflects the removal of inhibitors of the C1 complex assembly. The activity recovered from the Q Sepharose FF-2 chromatographic step was applied to a Mono Q column. In this stage, the C1 activity was recovered in two distinct elution peaks.
Purification of the C1 Factor

Fig. 2. Graphic representation of the chromatographic purification of the mammalian C1 factor. The chromatographic purification of the C1 factor is schematically illustrated in the top left panel. The remaining panels are graphic depictions of the elution of the total protein (solid line) and the total C1 activity (broken line) of each chromatographic step. The inset in each graph is a schematic representation of the applied millimolar KCl gradients (slanted lines) and step elutions (horizontal lines) that were used to develop the appropriate column. The approximate elution of the C1 factor activity is indicated with a horizontal bar. In the Mono Q panel, the elutions of pool I (PI) and pool II (PII) activities are designated. The Phosphocellulose, Q Sepharose, and Mono S panels are the representations of the phosphocellulose E, Q Sepharose FF-A, and Mono S pool I (MSD preparation) C1 factor activity fractionations.
**Assembly of the C1 complex with chromatographic fractions containing the C1 factor activity.** Protein-DNA binding reactions were done as described under "Materials and Methods" and contained 0.5 μl (Phosphocellulose, top panel) or 0.02 μl (Mono S, bottom panel) of the chromatographic fraction indicated at the top of each gel. Each panel represents that segment of the chromatographic fractionation that exhibits C1 factor activity. The position in the fractionation from which the C1 factor fractions were derived is indicated at the top of each panel by the concentration of KCl (mM). Broken lines indicate a position in the linear gradient of KCl, whereas the solid lines indicate a position in the isocratic elution. *Ld* and *FT* represent assays containing aliquots of the column load and flow-through fractions, respectively, --, control reaction in the absence of the C1 factor. The position of the Oct-1 POU-DNA and C1 complexes are indicated at the left of each panel. Only the top portion of each gel is shown, as the lower portions contain only the unbound HSVα0 DNA probe.

**Table I**

### Calculation of the activities and yields of the chromatographic purification of the mammalian C1 factor

The activities and chromatographic yields of the C1 factor preparations were calculated from the data obtained from quantitative C1 complex formation assays as described under "Materials and Methods." The total activity (in standard units), total protein (mg), and specific activity are listed for the material that was loaded onto the indicated chromatographic matrix (Load) and for the resulting chromatographic fractions containing the elution peak of the C1 activity (Elution Peak). The purification fold and % yield of the C1 activity is indicated for the individual (Stage) and the cumulative chromatographic stages (Cumulative). The first and second rows of the Mono Q data represent the calculations of the pool I and pool II C1 factors, respectively. The cumulative purification of the C1 factor polypeptide(s) is an underestimation due to the loss of C1 activity, but not total protein, during a technical problem encountered with the Mono Q HR 10/10 column. This loss has been incorporated into the cumulative purification and yield of the Q Sepharose FF-2 stage.

| No. | Column         | Load   | Elution peak | Stage | Cumulative |
|-----|----------------|--------|--------------|-------|------------|
|     |                | Activity | Protein | Specific activity | Activity | Protein | Specific activity | Purification Yield | Cumulative Yield |
| 1   | Phosphocellulose | 9,147.0 | 14,480.00 | 0.6 | 2,809.4 | 319.90 | 8.8 | 14.0 | 30.7 | 14 | 30.7 |
| 2   | Q Sepharose FF-1 | 2,809.4 | 319.90 | 8.8 | 3,043.0 | 24.40 | 124.7 | 14.2 | 108.0 | 99 | 35.1 |
| 3   | Q Sepharose FF-2 | 3,043.0 | 24.40 | 124.7 | 5,215.0 | 24.50 | 212.8 | 1.7 | 171.0 | 126 | 21.1 |
| 4   | Mono Q          | 1,580.0 | 19.80 | 79.8 | 2,608.0 | 1.92 | 1,358.0 | 17.0 | 165.0 | 2,142 | 34.8 |
|     | Mono S (MSD)    | 2,608.0 | 1.92 | 1,358.0 | 1,822.0 | 0.76 | 5,521.0 | 4.0 | 70.0 | 8,568 | 24.3 |
|     | Mono S (MSE)    | 710.0   | 0.97  | 728.0 | 286.5  | 0.18  | 3,768.0 | 5.1 | 40.0 | 5,850 | 3.8 |

As summarized in Table I, the total chromatographic fractionation resulted in an ~9000-fold cumulative purification of 24% of the C1 activity that was detected in the initial HeLa cell nuclear extracts. The cumulative yield is likely to be an overestimation of the actual yield of the C1 activity, since the association of the C1 factor with Oct-1 and “TIF appears to be partially inhibited in the nuclear extracts and the initial chromatographic fractions. However, the cumulative purification of the C1 factor polypeptide(s) is likely to be an underestimation (2–3-fold; refer to the legend to Table I). The described protocol (MSD and MSE preparations) resulted in the purification of ~235 pmol of the C1 factor.

**The Preparations of Purified C1 Activity Contain Multiple Polypeptides**—The fractions containing the peak of the C1 polypeptide(s) were each individually chromatographed in a Mono S column. In both cases, the highest concentration of C1 activity eluted at 100 mM KCl (Figs. 2 and 3, bottom). This final chromatographic step of the pool I (MSD) and II (MSE) activities resulted in a 4–5-fold purification of 70 and 40% of the applied activity, respectively (Table I).
activity from the various stages of this purification were analyzed by SDS-PAGE. Fig. 4 (left and center) illustrates the polypeptides present in these fractions from the phosphocellulose, Q Sepharose FF-2, Mono Q (pool I), and Mono S (MSD) chromatography. The purified C1 factor activity is represented by MSD fractions 9–13, which contain a polypeptide of 100 kDa and a cluster of three proteins of 123–135 kDa. Less abundant polypeptides of 68-, 180-, and 230-kDa proteins are also evident. Fig. 4 (right) shows the resolved polypeptides of the purified C1 activity from two independent fractionations, where MSA represents the pool I activity of preparation I, MSB is the pool II of preparation I, and MSD is the pool I of preparation II. Clearly, all of these fractions contain the 100- and 123–135-kDa polypeptides. In addition, the pool II C1 factor of both preparations contain significantly higher levels of the 68- and 180-kDa polypeptides, whereas the pool I fractions contain the 155- and 230-kDa proteins (Fig. 4, right, and data not shown).

The picomoles of the polypeptides in the C1 factor fractions were estimated from densitometric scans of silver-stained, SDS denaturing gels. The comparison of the picomoles of protein that were required to account for the amount of C1 complex activity indicated that the activity is mediated by one or more of the abundant polypeptides.

Requirements for the Formation of a C1 Complex—The C1 factor is defined as the activity that is required for the assembly of Oct-1 and αTIF into a specific multiprotein complex on the α/IE response element (65). To determine if the purified C1 polypeptide(s) retained the characteristics of the defined activity, MSD fraction 13 was added to a C1 complex formation reaction in the presence and absence of their ability to support the formation of a C1 complex in the presence of Oct-1 and αTIF. As shown in Fig. 6, the C1 factor eluted near the void volume of the column with a molecular mass that ranged from approximately 0.5–1.5 × 10⁶ Da. Furthermore, the heterogeneous chromatographic size of the C1 factor is visibly evident by the formation of a series of distinctly migrating C1 complexes in the gel shift assay (Fig. 6; compare fractions 26–30).

The Individual Polypeptides of the C1 Factor Preparations Do Not Contain C1 Activity—In an attempt to determine if a particular polypeptide in the purified C1 factor preparations was solely responsible for the activity, nuclear extracts of HeLa cells and C1 factor chromatographic fractions were precipitated, resuspended in 6 M urea, and renatured by dialysis. Under these conditions, 5% of the precipitated C1 activity could be recovered (data not shown). However, when the polypeptides of the purified factor were resolved by SDS-PAGE, eluted from the gel, and treated in a similar manner, no single polypeptide was capable of assembling Oct-1 and αTIF into a C1 complex (data not shown). It is possible that the recovery of C1 activity in the precipitated extracts was due to the presence of stable protein aggregates that rapidly renatured. However, due to the inefficient renaturation of the C1 factor and the limited sensitivity of the assay, it was not possible to test the hypothesis that the C1 activity was dependent upon the renaturation of specific combinations of SDS-PAGE-resolved polypeptides.

High Affinity Binding of the C1 Factor Polypeptides to

![Fig. 4. The polypeptides of the purified C1 factor activity. The left and center panels are parallel, silver-stained, 9.0% SDS-denaturing gels showing the resolution of the polypeptides in the peak C1 activity fractions of the phosphocellulose F (PC), Q Sepharose FF-A (QFF), Mono Q (MQ), and Mono S (pool I, MSD, fractions 9–13) chromatographic stages. PC, QFF, MQ, and MSD 11 each represent ~40 saturation units of C1 factor activity. The MSD column load (Ld) and fraction numbers are indicated at the top of the center panel and represent the electrophoresis of equivalent fraction volumes. The right panel depicts the resolution, in a 7.5% SDS denaturing gel, of the polypeptides in the purified C1 factor preparations as follows: MSA, preparation 1-pool I; MSB, preparation 1-pool II; and MSD, preparation II-pool I. The molecular weights, in thousands, of the protein markers (MW) are indicated at the left of each panel.](image)
αTIF — A significant characteristic of the C1 factor is its high affinity interaction with αTIF in the absence of the other C1 complex components (22, 71). To identify the polypeptide(s) that directly associate with αTIF, the purified C1 factor preparations were resolved by SDS-PAGE, transferred to a nitrocellulose filter, and incubated with [32P]αTIF protein. As illustrated in Fig. 7 (right panel), the αTIF probe specifically bound the 123-, 130-135-, and 230-kDa polypeptides in the active fraction from the initial phosphocellulose chromatography (PC). Similarly, in each of the purified C1 factor preparations (MSD, MSA, and MSB), αTIF bound the 123- and 130–135-kDa polypeptides. The protein additionally associated with the 155- and the 180-kDa proteins that are uniquely represented in the pool I (MSD and MSA) and pool II (MSB) preparations, respectively. In contrast, the 100-kDa polypeptide, common to all of the C1 factor pools, and the 68-kDa protein of the pool II preparation did not bind to αTIF in this assay (compare Fig. 7, left and right panels). The specificity of these interactions is evident from the lack of association of the labeled αTIF protein with the major polypeptides of the crude phosphocellulose chromatographic fraction (PC) and SDS-PAGE-resolved Escherichia coli lysates (Fig. 7 and data not shown).

The Interaction of the C1 Factor Polypeptides with αTIF Is Modulated by Their Phosphorylation State — Treatment of the C1 activity, in nuclear extracts or in chromatographic fractions, with potato acid phosphatase generated a C1 activity that formed a more rapidly migrating C1 complex, suggesting that one or more of the polypeptides of this factor are phosphoproteins (Ref. 22 and data not shown). Therefore, the purified C1 factor preparation was similarly treated with phosphatase, and the reaction was resolved by SDS-PAGE. In comparison with untreated fractions, treatment of the MSD preparation resulted in an alteration in the migration of the 100- and 123–135-kDa polypeptides (Fig. 7, left panel, MSD and MSD*).

To determine if the phosphorylation status of these polypeptides might affect their ability to interact with αTIF, the MSD fraction was treated with potato acid phosphatase and incubated with the αTIF protein probe. As shown in Fig. 7 (right panel, MSD and MSD*), this treatment eliminated the interaction of the 130–135- and 155-kDa polypeptides and significantly (2.7-fold) reduced the interaction of the 123-kDa protein with αTIF, compared with the untreated factor.

Partial Tryptic Digestion of the C1 Factor Polypeptides — The interaction of αTIF with multiple polypeptides (123–180 kDa) in the purified C1 factor preparations suggested that several of the polypeptides might be variant forms of a single protein. This possibility was addressed by comparing the patterns of tryptic peptides derived from the partial digest of the 68-, 100-, and 123–135-kDa proteins (Fig. 8). The fractions containing the purified C1 factor were resolved by SDS-PAGE and transferred to nitrocellulose. Under these gel conditions, three major protein bands (68-, 100-, and the 123–135-kDa proteins (Fig. 4, MSD fraction 13)) were detected after staining the membrane with Ponceau S. The excised protein bands were incubated with trypsin, and the eluted peptides were resolved by HPLC reverse phase chromatography.

Fig. 8 depicts the elution profile of the resolved peptides of the 123–135-kDa (top panel) and the 100-kDa (bottom panel) proteins. Although the individual polypeptides of the 123–135-kDa band were not resolved, and were, therefore treated as a single sample, the pattern of tryptic peptides was that expected of a relatively homogeneous sample, with no indi-
purification of the C1 Factor—The C1 factor is required for the stable assembly of αTIF and the Oct-1 homeodomain on the HSV α/IE element. Previous characterization of the C1 component had indicated that it was an evolutionarily conserved factor, being found in both insect and human cells (65). The factor did not independently bind the α/IE enhancer element but interacted directly with αTIF to form an αTIF-C1 factor protein complex. This protein complex is probably responsible for the stable recognition of the Oct-1-α/IE element complex.

The development of a scheme for the purification of the C1 factor from mammalian cell extracts required consideration of the nonhomogeneous chromatographic behavior of this protein(s). This observation suggested that the protein might be composed of multiple subunits and/or be associated with a heterogeneous group of proteins in the nuclear extracts. Therefore, the factor was chromatographically purified in the presence of 3 M urea, which resulted in a consistent and homogeneous fractionation. Approximately 235 pmol of C1 factor was thus purified ~9000-fold from nuclear extracts of ~1 × 10^11 HeLa cells, with a final yield of ~25% of the initial C1 factor activity. Thus, the C1 factor is present at approximately 5000 molecules/cell.

The purified C1 factor preparations contained a set of polypeptides of 100- and 123–135-kDa, with less abundant species of 68- and 155-kDa. In later stages of the purification, the factor eluted from the Mono Q column in two chromatographically distinct peaks of activity: pool I and pool II. Both of these pools contained the 100- and 123–135-kDa polypeptides. In addition, the various C1 factor preparations contained only these polypeptides in a sufficient stoichiometric quantity to account for the C1 activity. Therefore, it is likely that the C1 factor is composed of one or more of these proteins.

The C1 Factor Is Likely to Be a Multiprotein Complex of Polypeptides of 100- and 123–135-kDa—Several experiments strongly suggest that the polypeptides present in the various C1 factor preparations represent the tightly associated components of a multiprotein C1 factor. First, the cofractionation of these proteins in the presence of 3 M urea is unusual. Second, gel filtration chromatography of the C1 factor under relatively stringent conditions indicates that the factor has a molecular mass of approximately 0.5–1.5 × 10^6 Da. Third, although an interpretation of a negative result, it was not possible to recover C1 activity by renaturation of any single polypeptide in the purified C1 factor preparations. Finally, the tryptic peptide analysis indicated that the 100-kDa polypeptide is present at a 1:1 stoichiometric ratio with the set of 123–135-kDa proteins in the purified C1 factor preparations. Collectively, the results are consistent with the hypothesis that the C1 factor is composed of multiple subunits.

Characterization of the purified C1 factor further suggests that it is heterogeneous. As detected in the gel shift assay, the C1 complex band is actually a set of tightly migrating com-

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phorylation of the ~62~ polypeptide, apparently by regulat-
post-translational modification is reminiscent of the regulated
dependent phosphorylation of the Rb protein (83; for reviews,
see Refs. 84 and 85). Similarly, the formation of the ternary
peptides of the C1 factor preparations demonstrates that the
factor polypeptides may be a critical point in the regulation
of this interaction. Thus, the phosphorylation status of the C1
factor polypeptide(s), 
proteins are present in an approximate 1:1 stoichiometric
ratio in the purified C1 factor preparations. These data sug-
get that the C1 factor consists of a complex of two related
subunits: the 100-kDa, in combination with the various forms
of the 123–135-kDa polypeptides.

The Interaction of the C1 Factor Polypeptides with αTIF: Regulation by Posttranslational Modification—The most sig-
ificant functional characteristic of the C1 factor is its high
affinity interaction with αTIF in the absence of the other com-
ponents of the C1 complex. This interaction could ac-
count for the role of the C1 factor in the C1 complex by
stabilizing a conformation of αTIF that would bind with a
higher affinity to the Oct-1-DNA complex. It is also possible
that the C1 factor interacts directly with the Oct-1 protein
and/or DNA in the context of the assembled complex. Ad-
ditional cellular factors have also been identified that appear
to associate with the C1 complex (C2 factor) but have not yet
been purified or characterized (65). Thus, the C1 complex
is probably part of a larger complex in the cell.

Analysis of the interaction of αTIF with the resolved polypep-
dides of the C1 factor preparations demonstrates that the
123–135-kDa polypeptides, but not the 100- and 68-kDa polye-
dides, specifically bind to the protein probe. Thus, the 123–
135-kDa species present in all of the C1 factor preparations,
probably part of a larger complex in the cell.

The distinct mobilities of these complexes is partially a result
of the phosphorylation status of the αTIF protein (84, 85). The
modulation of the affinity of this interaction may
play a role in determining the stability of the C1 complex.
Although alternative mechanisms may exist to regu-
late HSV αTIF gene expression (87), the regulated assembly
of this complex may be significant for the activation of
the lytic cycle of HSV gene expression.

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