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Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus

(Ni^{2+}-chelate affinity chromatography/serum response factor/*in vitro* transcription)

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ABSTRACT **Vaccinia virus has been used as a vector to express foreign genes for the production of functional and posttranslationally modified proteins. A procedure is described here that allows the rapid native purification of vaccinia-expressed proteins fused to an amino-terminal tag of six histidines. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+}·nitrilotriacetic acid (Ni^{2+}·NTA)-agarose and histidine-tagged proteins are selectively eluted with imidazole-containing buffers. In the case of the human serum response factor (SRF), a transcription factor involved in the regulation of the c-*fos* protooncogene, the vaccinia-expressed histidine-tagged SRF (SRF-6His) could be purified solely by this step to >95% purity. SRF-6His was shown to resemble authentic SRF by functional criteria: it was transported to the nucleus, bound specifically the c-*fos* serum response element, interacted with the $p62^{TCF}$ protein to form a ternary complex, and stimulated *in vitro* transcription from the serum response element. Thus, the combination of vaccinia virus expression and affinity purification by Ni^{2+}·NTA chromatography promises to be useful for the production of proteins in a functional and posttranslationally modified form.**

An important application of gene technology is the overproduction of different proteins that can be utilized as pharmaceutical agents, as antigens for the production of antibodies, or as tools for structural and functional analyses (1). Some of the prerequisites to obtain functional overproduced protein are correct folding, posttranslational modifications, and solubility. Not all of these criteria are met when eukaryotic proteins are produced in bacterial expression systems (2). Therefore, viral expression systems have been developed using eukaryotic cells as hosts (3, 4). One of the most versatile expression systems employs a member of the poxvirus family, the vaccinia virus. Extensive knowledge about the physiology as well as the regulation of vaccinia gene expression has helped in the design of vaccinia expression systems (5), in which a cDNA is most often fused to a vaccinia promoter and inserted into the viral genome via homologous recombination. Recombinant vaccinia viruses can then be used for the production of soluble, posttranslationally modified proteins in a multitude of animal and human cells (6, 7). One drawback of the vaccinia system, in comparison to bacterial ones, is the lower level of expression necessitating extensive purification to obtain pure protein.

To facilitate rapid and efficient purification we have designed a vaccinia recombination vector that directs the expression of foreign proteins with an additional amino-terminal affinity tag of six histidine residues. This affinity tag has previously been used to facilitate purification of fusion proteins expressed in bacteria by employing a Ni^{2+}·nitrilotriacetic acid (Ni^{2+}·NTA) affinity column under denaturing conditions (8, 9). We have developed a procedure allowing the purification of histidine-tagged proteins under native conditions. This procedure is rapid, involves only one chromatographic step, and results in the isolation of highly enriched functional protein. Here this approach is applied to the expression and purification of the human serum response factor (SRF), which is a nuclear transcription factor participating in the regulation of the c-*fos* protooncogene (10, 11). SRF is posttranslationally modified by glycosylation (12) and phosphorylation (13), the latter being apparently necessary for SRF activities (14). These include specific DNA binding, protein–protein interaction, and activation of transcription (10, 15). We demonstrate that the vaccinia-expressed histidine-tagged SRF protein exerts the same activities as described for SRF.

MATERIALS AND METHODS

Construction of Vaccinia Recombination Plasmid pSRF-6His. pSRF-6His was constructed by inserting SRF cDNA between the blunted *Sph* I site and the *Bam*HI site of the vaccinia recombination vector pMS-56. The SRF cDNA insert was isolated from pT3G-SRF (12), which was first cut with *Bgl* I, followed by S1 nuclease resection of the overhanging ends, and then with *Bam*HI.

Generation of Recombinant Vaccinia Virus SRF-6His. An 80% confluent monolayer of human osteosarcoma 143 B cells (35-mm dish) was infected with 0.1 plaque-forming unit of vaccinia virus per cell. After 2 hr at 37°C, cells were transfected with 500 ng of pSRF-6His DNA by the calcium phosphate coprecipitation method (4). Viruses were harvested 2 days later and recombinants were selected in medium containing 25 μg of mycophenolic acid per ml, 250 μg of xanthine per ml, and 15 μg of hypoxanthine per ml using rabbit kidney RK_{13} cells (16). Single recombinant viruses were then isolated by one plaque purification step in the same selection medium and then further selected with 5-bromodeoxyuridine on thymidine kinase-deficient human osteosarcoma 143 B cells (4).

Purification of Native and Denatured SRF-6His Protein. HeLa monolayer cells (eight dishes, 15-cm diameter) were infected at a multiplicity of infection of 2 with SRF-6His vaccinia virus and incubated for 18 hr at 37°C. Cells were then washed with physiological phosphate buffer, detached from the dishes by incubating in 40 mM Tris·HCl, pH 7.5/10 mM EDTA/150 mM NaCl for 5 min, and collected by centrifugation (1000 × *g*, 5 min). All further steps were performed at 4°C.

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Abbreviations: NTA, nitrilotriacetic acid; SRF, serum response factor; SRE, serum response element.

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Cells were resuspended in 4.5 ml of 10 mM Hepes, pH 7.9/5 mM $MgCl_2$/0.1 mM EDTA/10 mM NaCl/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride/10 mM NaF (buffer A). After 5 min of swelling, cells were lysed in a Dounce homogenizer (pestle B, 20–40 strokes). The lysate was mixed with 1 volume of buffer A/830 mM NaCl/34% glycerol/1.6 mM imidazole and incubated for 45 min with continuous agitation. Cell debris and nuclei were pelleted (10,000 × *g*, 10 min) and the supernatant was incubated with 0.5 ml of Ni^{2+}·NTA-agarose (Qiagen, Chatsworth, CA) for 1 hr with continuous agitation. The resin was packed into a column, washed once with 4.5 ml of 10 mM Hepes, pH 7.9/5 mM $MgCl_2$/0.1 mM EDTA/50 mM NaCl/1 mM dithiothreitol/17% glycerol (buffer D) supplemented with 0.1 mM phenylmethylsulfonyl fluoride/10 mM NaF/0.8 mM imidazole, and then washed twice with 4.5 ml of the previous buffer with 8 mM imidazole. After two washes with 4.5-ml volumes of buffer D/40 mM imidazole, the SRF-6His protein was eluted with two 4.5-ml volumes of buffer D/80 mM imidazole. Protein fractions were frozen in liquid N_2 and stored at −70°C.

Purification of denatured SRF-6His protein was done essentially as described (9) with the addition of a dithiothreitol/4-vinylpyridine treatment and reverse-phase chromatography on a Pro-RPC HR5/2 column (Pharmacia) applying a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. Purified denatured SRF-6His protein was stored lyophilized at −20°C.

$NaDodSO_4$/PAGE, Immunoblotting, and Cell Staining. $NaDodSO_4$/PAGE, silver staining, and immunoblotting using alkaline phosphatase-coupled second antibodies were as described (17). For intracellular localization of SRF-6His protein, HeLa cells grown on a coverslip were fixed with methanol 12 hr after infection with recombinant SRF-6His vaccinia virus. Fixed cells were processed for immunofluorescent staining using rhodamine-coupled second antibodies and for DNA staining with the Hoechst 33258 dye according to standard procedures (17).

Mobility-Shift and *in Vitro* Transcription Assays. The mobility-shift assays were carried out essentially as described (18). The ^{32}P end-labeled probe and the specific competitor spanned the c-*fos* promoter from −330 to −278, which contains the

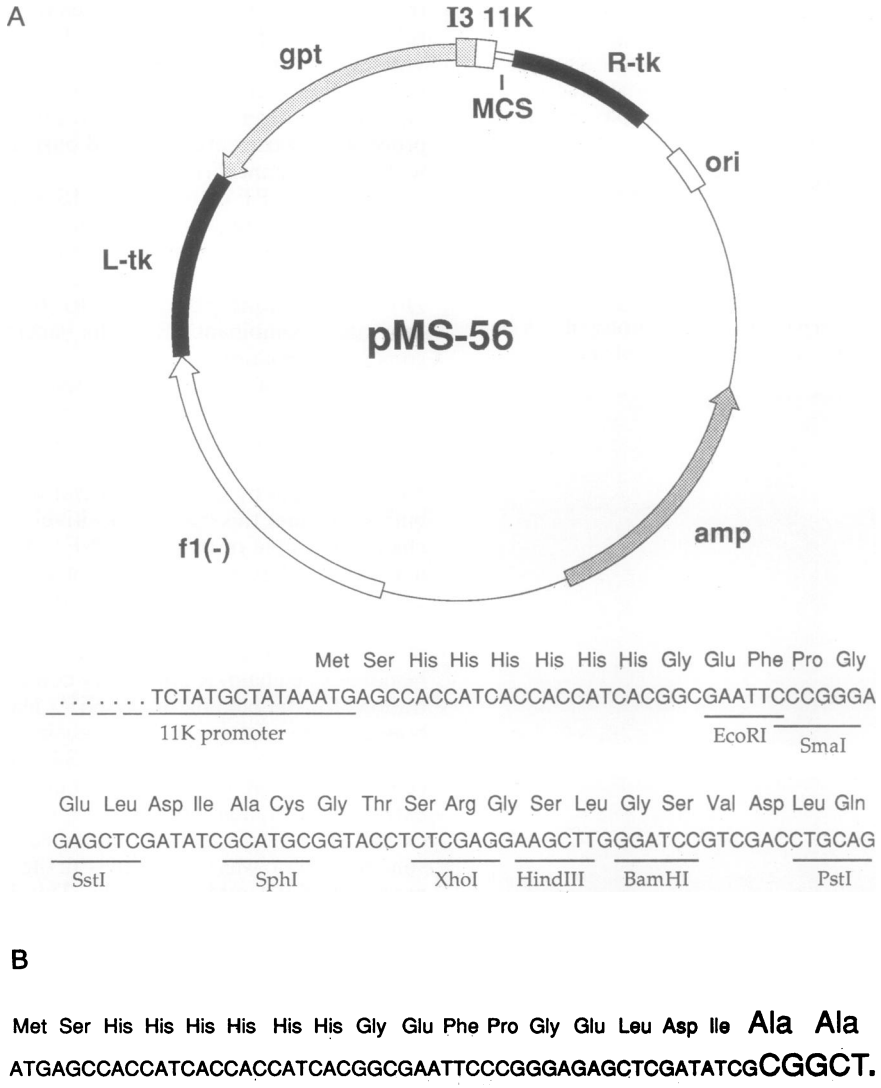

FIG. 1. Cloning of pSRF-6His. (*A*) Structure of the vaccinia virus recombination vector pMS-56. The DNA sequences of the histidine-tag coding region and of the multiple cloning site (MCS) are depicted. Only unique restriction sites within the MCS are shown. ori, Bacterial origin of replication; amp, ampicillin-resistance gene; f1(−), f1 phage-origin of replication; gpt, *Escherichia coli* xanthine guanine phosphoribosyltransferase gene; L-tk and R-tk, left and right parts of the vaccinia thymidine kinase gene; I3 and 11K, vaccinia promoters. (*B*) DNA and deduced protein sequence of the amino terminus of the SRF-6His fusion protein. The first 17 amino acids of the fusion protein, which comprised the tag of six histidines, were derived from pMS-56 sequences and fused to the SRF protein devoid of its first 9 amino acids. Larger letters indicate sequences derived from SRF cDNA.

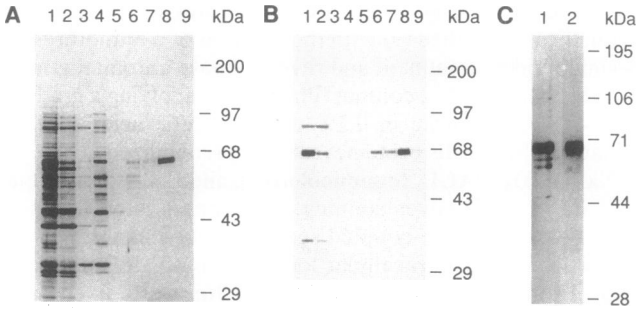

FIG. 2. Purification of SRF-6His protein. (*A*) HeLa cells were infected with recombinant SRF-6His vaccinia virus and a whole cell extract was prepared. SRF-6His protein was purified by native chromatography on a Ni^{2+}·NTA column. Proteins were visualized on $NaDodSO_4$ gels by silver staining. Lanes: 1, column load; 2, flow-through; 3, wash with 0.8 mM imidazole; 4 and 5, wash with 8 mM imidazole; 6 and 7, wash with 40 mM imidazole; 8 and 9, elution with 80 mM imidazole. Equal volumes of each fraction were electrophoresed. (*B*) Corresponding Western blot using anti-SRF antibodies. (*C*) HeLa cells infected with recombinant SRF-6His vaccinia virus were lysed in 6 M guanidine (pH 8.0) buffer and SRF-6His protein was purified by nonnative chromatography on a Ni^{2+}·NTA column. The pH 4.0 eluate from this column was further purified by reverse-phase chromatography. Proteins were visualized on $NaDodSO_4$ gels by silver staining. Lanes: 1, pH 4.0 eluate; 2, peak fraction of SRF-6His protein from reverse-phase chromatography eluting at 31–33% acetonitrile.

serum response element (SRE). The nonspecific competitor corresponded to the direct repeats in the c-*fos* proximal promoter domain (18). Native SRF was purified to homogeneity by DNA-affinity and wheat-germ agglutinin chromatography (12). $p62^{TCF}$ was partially purified according to Schröter *et al.* (12). Details of the *in vitro* transcription protocol will be described elsewhere (R.A.H. and A.N., unpublished data).

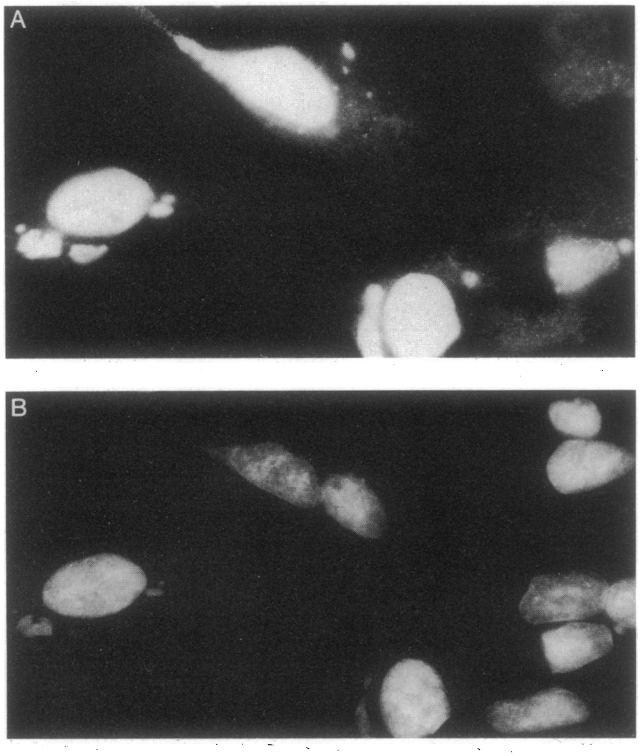

FIG. 3. Subcellular localization of the SRF-6His protein. (*A*) Immunofluorescent staining with anti-SRF antibodies of HeLa cells infected with recombinant SRF-6His vaccinia virus at a multiplicity of infection of 0.5. (*B*) Corresponding staining of DNA with the Hoechst 33258 dye. (×500.)

Transcription was performed in HeLa nuclear extract depleted of SRF using a SRE-affinity resin. This was complemented with SRF-6His or native SRF and various promoters fused to different-sized G-free cassettes, as indicated.

RESULTS

pMS-56 and the Generation of Recombinant SRF-6His Vaccinia Virus. The vaccinia recombination plasmid pMS-56 (Fig. 1*A*) was designed to express proteins as fusion products containing an additional amino-terminal stretch of six histidines. Unique restriction sites present downstream of the six-histidine coding region allow the cloning of cDNA in frame with the histidine tag. Transcription of the resulting fusion gene is under the control of the strong vaccinia virus 11K late promoter (19). Stable integration of the cDNA into the vaccinia virus genome is mediated by flanking sequences, which are homologous to the vaccinia virus thymidine kinase locus (Fig. 1*A*). pMS-56 also contains a chimeric gene consisting of the vaccinia virus I3 intermediate promoter (20) and the *E. coli* xanthine guanine phosphoribosyltransferase gene that is cointegrated with the cDNA into the viral genome and thus can serve as a dominant positive marker to select for recombinant viruses (16). Copurification of some wild-type viruses does occur, but wild-type viruses can be eliminated by selection with 5-bromodeoxyuridine using thymidine kinase-deficient cells (4). In the presence of this agent, viable progenitor viruses are produced only in cells infected solely with recombinant virus.

We cloned SRF cDNA into pMS-56 in such a way that the resulting fusion protein SRF-6His contained 17 new amino acids at its amino terminus, including the six histidines. This effectively replaced the first nine amino acids of SRF (Fig. 1*B*). The resulting plasmid, pSRF-6His, was then used to generate recombinant SRF-6His vaccinia virus via homologous recombination.

Purification of Vaccinia-Expressed SRF-6His Protein. It has been shown that proteins fused to a stretch of polyhistidine bind to Ni^{2+}·NTA resin under denaturing conditions (8, 9). These fusion proteins can then be eluted from the Ni^{2+}·NTA affinity column by drastically lowering the pH of the column buffer, because this leads to positively charged histidine side chains incapable of binding to Ni^{2+}. We have used an alternative procedure that avoids protein denaturation. Proteins can be bound to the Ni^{2+}·NTA affinity column under native conditions in low or high salt buffers and eluted by application of imidazole. This molecule has the same structure as the histidine side chain. It effectively competes at higher concentrations for the binding to the Ni^{2+}, leading to the elution of bound fusion proteins without causing denaturation.

For the native purification of SRF-6His protein, infected cells were lysed in hypotonic buffer and the lysate was extracted with high salt buffer. The resulting whole cell extract (Fig. 2*A*), which contained SRF-6His at ≈1% of the soluble protein, was incubated with the Ni^{2+}·NTA resin in the presence of 0.8 mM imidazole. This low concentration of imidazole reduced nonspecific binding, which could be suppressed even more by further increasing the concentration of imidazole to 8 mM. However, this also led to a larger proportion of SRF-6His protein present in the unbound fraction (data not shown).

The Ni^{2+}·NTA resin was washed with 8 mM imidazole, which removed a substantial fraction of the nonspecifically bound protein (Fig. 2*A*). Washing with 40 mM imidazole resulted in the further elimination of contaminants but also in the elution of some SRF-6His protein (Fig. 2*B*). The vast majority of SRF-6His was eluted at 80 mM imidazole. The SRF-6His in lane 8 of Fig. 2*A* was >95% pure as estimated from the silver staining of the protein gel, which indicated an ≈100-fold purification. Furthermore, the SRF-6His con-

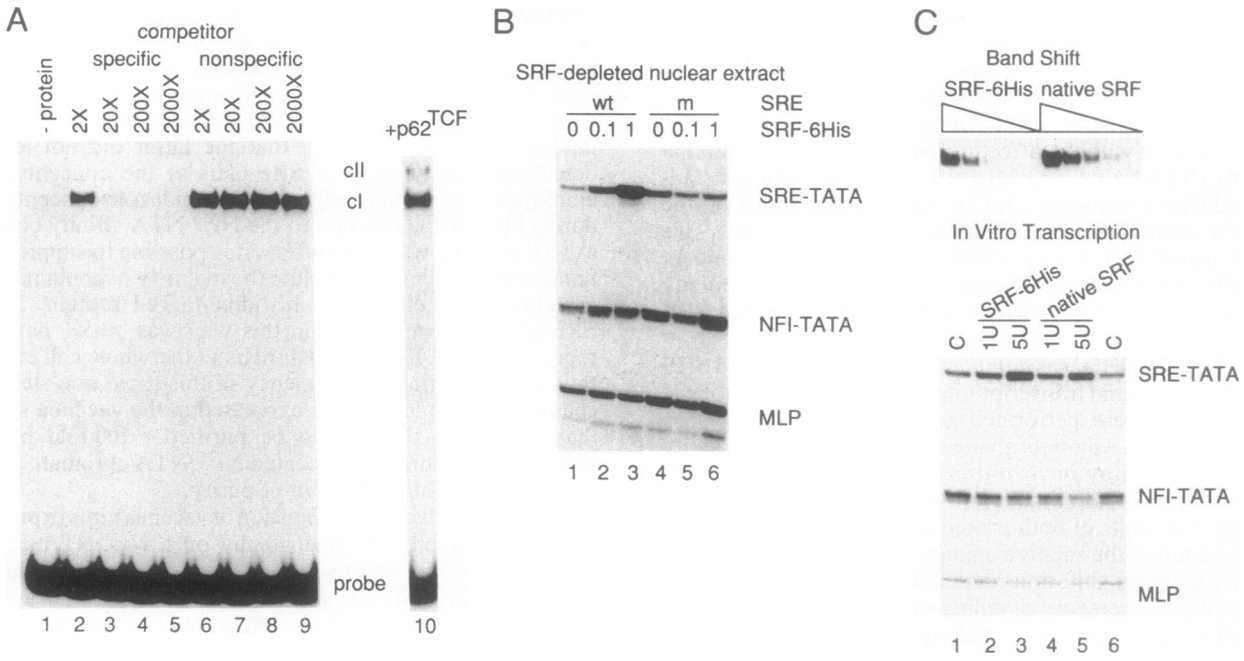

FIG. 4. Functional analysis of the activity of purified native SRF-6His. (*A*) Autoradiography of a mobility-shift gel. Lane 1, no added SRF-6His. Lanes 2–9, SRF-6His was preincubated with increasing amounts (as indicated above each lane) of either a SRE-specific competitor (lanes 2–5) or a nonspecific competitor (lanes 6–9), after which the ^{32}P-labeled c-*fos* SRE was added and the incubation was continued. Lane 10, SRF-6His and partially purified p62^{TCF} were added together with the probe. (*B*) Autoradiography of a sequencing gel showing *in vitro* transcripts generated in SRF-depleted HeLa nuclear extract. All reaction mixtures contained two different G-free cassettes driven by a nuclear factor I binding site fused to the c-*fos* TATA box (NFI-TATA) or the adenovirus major late promoter (MLP). Lanes 1–3, wild-type (wt) SRE fused to a TATA box/G-free cassette was included (SRE-TATA); lanes 4–6, the same construct but with a mutated (m) SRE. Lanes 1 and 4, no added SRF-6His; lanes 2 and 5, 0.1 μl of purified native SRF-6His added; lanes 3 and 6, addition of 1 μl of SRF-6His. (*C*) Comparison of DNA-binding and *in vitro* transcription activities of SRF-6His and native purified SRF. The upper panel shows the formation of complex I (cI) as in *A* by a series of five dilutions of both proteins. The second and the eighth lane correspond to one mobility-shift unit of each protein. The lower panel shows the *in vitro* transcriptional activity as in *B* of one and five mobility-shift units of SRF-6His and native purified SRF. Lanes 1 and 6, no added SRF-6His or SRF.

tained in this fraction was >50% of the SRF-6His protein present in the whole cell extract as judged from the Western blot (see Fig. 2*B*). The apparent molecular mass of the SRF-6His protein was in agreement with that observed for SRF in nuclear extracts prepared from HeLa cells (≈67 kDa).

To compare the native purification procedure to the non-native one, HeLa cells infected with SRF-6His vaccinia virus were lysed in a pH 8.0 buffer with 6 M guanidine. The resulting whole cell lysate was incubated with Ni^{2+}·NTA resin. Bound protein was eluted at pH 4.0 after washing with 6 M guanidine buffer of pH 5.9 (9). The eluted SRF-6His protein (Fig. 2*C*) was not as pure as native SRF-6His eluted with imidazole. To obtain an equivalent purity, the denatured SRF-6His protein had to be further purified by reverse-phase chromatography. Fig. 2*C* shows that denatured SRF-6His protein was >95% pure after reverse-phase chromatography.

Functional Characterization of Vaccinia-Expressed SRF-6His Protein. The SRF protein is a nuclear transcription factor (11), which forms two specific complexes with a DNA fragment containing the c-*fos* SRE. Complex I contains a dimer of SRF, and complex II additionally contains the protein p62^{TCF} (15). Furthermore, the SRF protein constitutively activates transcription *in vitro* (10). We tested the vaccinia-produced SRF-6His protein to determine whether it exhibited the same functional characteristics as native SRF protein.

To investigate the subcellular localization of SRF-6His protein, HeLa cells were infected with recombinant SRF-6His vaccinia virus at a multiplicity of infection of 0.5 and the SRF-6His protein was localized by immunofluorescent staining of the cells using anti-SRF antibodies (Fig. 3). Cells were also stained with Hoechst dye, which is indicative of the presence of DNA and thus stained the cell nuclei and the cytoplasmatic viral factories (21). This allowed identification of cells infected with virus. Immunofluorescent staining was observed in nuclei of cells infected with SRF-6His vaccinia virus. Nuclei of noninfected cells were not stained at the employed concentrations of anti-SRF antibodies, indicating that endogenous SRF was present at significantly lower concentrations in HeLa cells than SRF-6His in infected HeLa cells. The immunofluorescent staining in the cytoplasm of infected cells was indistinguishable from that of noninfected cells, except for the positive staining of viral factories. HeLa cells infected with wild-type vaccinia virus showed no significant SRF immunofluorescent staining of cell nuclei and of viral factories (data not shown). These results demonstrate the nuclear localization of SRF-6His as well as its presence within the viral factories.

Native purified SRF-6His protein (Fig. 2*A*, lane 8) was tested in two functional assays, as shown in Fig. 4. It formed a complex with the human c-*fos* SRE (cI, Fig. 4*A*), the specificity of which was demonstrated by competition with an oligonucleotide containing the SRE. Complex I formation was greatly reduced by a 20-fold molar excess of the unlabeled competitor (Fig. 4*A*, lane 3) and a further excess completely eliminated it (lanes 4 and 5). In contrast, even a 2000-fold excess of a nonspecific oligonucleotide did not significantly affect complex I formation (lane 9). Furthermore, SRF-6His was able to form a ternary complex with p62^{TCF} (cII, lane 10), as described for SRF purified from HeLa cell nuclear extracts (15). Thus SRF-6His behaved identically to purified SRF from HeLa cells in mobility-shift assays.

We then tested whether SRF-6His also stimulated *in vitro* transcription in a SRE-dependent manner (Fig. 4*B*). Two templates containing SREs fused to the c-*fos* TATA box and a G-free cassette were tested, one with a wild-type SRE (lanes 1–3) and the other with a mutated SRE (lanes 4–6) that cannot bind SRF or compete for SRF binding to a wild-type

SRE (15). Both templates showed similar levels of transcription without added SRF-6His (SRE-TATA, lanes 1 and 4). However, adding increasing amounts of purified native SRF-6His led to up to 10-fold elevated levels of transcription from the template containing the wild-type SRE (SRE-TATA, lanes 2 and 3) without affecting either of the two internal controls (NFI-TATA and MLP). In contrast, SRF-6His addition did not stimulate the *in vitro* transcription of the template containing the mutated SRE (lanes 4–6). This demonstrated that the stimulation *in vitro* was dependent upon SRF-6His and a wild-type SRE. The same specificity has been observed for purified and *in vitro* translated SRF (ref. 10; R.A.H., unpublished observations).

Finally, SRF-6His was compared with native purified SRF in DNA-binding and transcriptional assays. Serial dilutions of both proteins were performed and tested in mobility-shift assays (Fig. 4*C*). Amounts giving equal mobility-shift activity were then tested by *in vitro* transcription (Fig. 4*C*). Equivalent transcriptional stimulation was seen with comparable mobility-shift units of both proteins. By Western blotting we also determined the relative amount of SRF-6His and native SRF per mobility-shift unit (data not shown). This indicated that to obtain the same mobility-shift activity 2.5-fold less SRF-6His was required as compared with native purified SRF. The higher specific activity of SRF-6His could be attributed to the rapidity of its purification, within hours, instead of the several days required to obtain homogeneous native SRF protein.

DISCUSSION

We have developed a procedure based on Ni^{2+}·NTA affinity chromatography that allows the rapid and native purification of vaccinia virus-expressed proteins tagged with six histidines. The value of this native purification procedure was demonstrated with the vaccinia-expressed SRF-6His protein and several other transcription factors, including Zn^{2+}-finger proteins (G.d.M. and H.G.S., unpublished). Analysis of the SRF-6His protein has shown that posttranslational glycosylation and phosphorylation events known to occur on native SRF protein (12, 13) were also observable for the vaccinia-expressed SRF-6His (data not shown). By several criteria, native SRF-6His possessed the same functional activities as SRF purified from HeLa cell nuclear extracts: binding to the c-*fos* SRE, interacting with $p62^{TCF}$ to form a ternary complex on the SRE, and stimulating transcription *in vitro* in a SRE-dependent manner. Additionally, the SRF-6His protein was shown to be translocated to the nucleus in cells infected with recombinant virus. Thus, the function of vaccinia-expressed SRF-6His does not seem to be affected by the histidine tag.

Previously, histidine-tagged proteins were purified by Ni^{2+}·NTA chromatography under denaturing conditions (8, 9). The advantages of the nonnative purification procedure are that enzymes, such as proteases or phosphatases, are instantly inactivated upon cell lysis in 6 M guanidine, a fact of utmost importance when investigating posttranslational modifications. Furthermore, denaturation might be necessary to free the affinity tag if it is buried inside the protein. Such a problem may be circumvented by fusing the affinity tag to the carboxyl terminus of the expressed protein, which will also be useful when expressing secreted proteins. The clear disadvantages of the nonnative purification procedure are that proteins are not retained in their native form and denaturation allows nonneighboring histidines to accommodate binding to the Ni^{2+}. This has probably caused the higher level of coeluting contaminating proteins observed for SRF-6His eluted from the Ni^{2+}·NTA resin under denaturing conditions than for SRF-6His eluted under native conditions (see Fig. 2).

To overcome these problems, proteins can be bound to the Ni^{2+}·NTA resin in a native state. Elution can be facilitated by lowering the pH, which often leads to denaturation, by high concentrations of chelating agents, which render many metal-containing proteins irreversibly inactive, or by means of imidazole. We have shown that the latter did not lead to detectable denaturation of SRF-6His at the concentrations employed. By carefully adjusting the imidazole concentration during the binding reaction to the Ni^{2+}·NTA affinity column, as well as in the washing buffers, it is possible to suppress the nonspecific binding and to elute the majority of contaminating proteins before eluting the histidine-tagged protein. Native SRF-6His protein purified in this way was >95% pure and represented >50% of the SRF-6His in the whole cell extract, thus demonstrating the efficiency of this rapid one-step procedure. Proteins less highly expressed in the vaccinia system than SRF-6His should also be purified ≈100-fold by this one-step procedure, and repeated Ni^{2+}·NTA chromatography might lead to a higher degree of purity.

We conclude that the combination of vaccinia virus expression system and affinity chromatography on a Ni^{2+}·NTA column offers a convenient means to rapidly purify large quantities of various proteins and mutagenized derivatives thereof in a native and functional state. These highly purified proteins may help in the determination of protein function and structure.

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