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Purification and Characterization of the Stage-Specific Embryonic Enhancer-Binding Protein SSAP-1

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We have demonstrated that a highly conserved segment of DNA between positions −288 and −317 (upstream sequence element IV [USE IV]) is largely responsible for the transcriptional activation of the sea urchin H1-β histone gene during the blastula stage of embryogenesis. This sequence is capable of acting as an embryonic enhancer element, activating target genes in a stage-specific manner. Nuclear extracts prepared from developmentally-staged organisms before and after the gene is activated all contain a factor which specifically binds to the enhancer. We have purified a 43-kDa polypeptide which binds to and footprints the USE IV enhancer element. We refer to this protein as stage-specific activator protein 1 (SSAP-1). Early in development before the enhancer is active, SSAP appears as a 43-kDa monomer, but it undergoes a change in its molecular weight beginning at about 12 h postfertilization (early blastula) which precisely parallels the increase in H1-β gene expression. Modified SSAP has an apparent molecular mass of approximately 90 to 100 kDa and contains at least one 43-kDa SSAP polypeptide. Thus, it is the disappearance of the 43-kDa species and the appearance of the 90- to 100-kDa species which coincide with the H1-β gene activation. The correlation between the change in molecular weight of SSAP and the stage-specific activation of H1-β gene expression strongly suggests that this higher-molecular-weight form of SSAP is directly responsible for the blastula stage-specific transcriptional activation of the late H1 gene.

A critical parameter governing normal development is the correct temporal and spatial regulation of gene expression. The sea urchin has several families of histone genes that are expressed at specific intervals in the developing embryo, making it an ideal system in which to study temporal gene regulation during early embryogenesis. The early histone genes are present in approximately 300 to 500 tandemly repeating units (for a review, see reference 40). Each tandem array consists of five independent transcription units, one for each histone class. Early histone gene synthesis begins shortly after fertilization and increases dramatically between the 16- and the 200-cell stages (approximately 6 to 12 h postfertilization), reaching a maximum of about 7×10^6 to 10×10^6 molecules per embryo for the core histone genes and about 5×10^6 for the early H1 genes (39, 43, 58). At this stage in development, the histone message constitutes as much as 50% of the total mRNA. During the subsequent cell divisions, both early histone gene transcription and histone mRNA abundance undergo an abrupt decline (6, 42, 58). These genes remain repressed for the rest of the life of the organism, except during oogenesis (29, 38).

The late histone genes are encoded by a smaller number of genes. The core histone genes are dispersed in the genome in 5 to 10 irregular arrays, while the two late H1 subtypes, H1-β and H1-γ, are encoded as unique copy genes (7, 28, 29, 32, 35, 41). The late histone genes are transcribed at low basal levels until the mid-blastula stage (12 to 18 h, 128 to 300 cells), after which the transcription rate increases and transcripts accumulate to a peak of about 2×10^6 molecules in the late-blastula stage embryo (21 h) (25, 30). The late histone genes remain active throughout the life of the organism and are the somatic set of histone genes. The accumulation of late histone transcripts coincides roughly with the decline in the early histone mRNA levels. This dramatic switch in expression from early to the late histone gene families provides us with an elegant system in which to study the mechanisms of temporal regulation during the early stages of embryonic development.

The ability of cloned genes to be properly regulated after microinjection into sea urchin eggs has permitted the dissection of the *cis*-regulatory regions controlling gene expression (8, 44). Microinjected copies of the CyIIIa actin gene, for example, are expressed only in the aboral ectoderm (24). Proper localization has been demonstrated also with the actin CyIa gene (27) and the Spec gene families (17, 54). Proper temporal regulation has been repeatedly demonstrated with the H3 (14), H2a (11, 14), H2b (9, 57), and H1 (14b) early histone genes, as well as the H2b (9) and H1 (37) late histone genes. We have shown that upon microinjection of early H3 DNA into *Litechinus pictus* one-cell zygotes transcripts begin to accumulate at 6 h postfertilization, reach a peak in 10- to 12-h embryos, and then decline, precisely paralleling the endogenous early H3 gene in the same embryos (14). Microinjected H1-β DNA, however, begins to be transcribed at 8 h postfertilization, reaching its highest level at about 20 h, again paralleling the endogenous late H1 gene in the same embryo (37).

The late histone genes have about 80% homology in their coding regions and also contain long stretches of near 100% homology in their 5′ upstream regions (31, 37). These sequences are not only shared by the two late H1 genes in *Strongylocentrotus purpuratus* but are also found the late H1-γ gene of *L. pictus*. This conservation seems striking, since these two species last shared a common ancestor between 30 million to 40 million years ago (53) and most of the unique sequence DNA in each is highly divergent. Four of these conserved sequences (the TATA box, upstream sequence element 0 [USE 0], USE I, and USE II) are also shared with the H1 early gene, while two sequences (USE

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III and USE IV) are late gene specific (37). Of the two late gene-specific sequences, element IV seems to play the major role in the blastula stage activation of the H1-β gene. A 30-bp internal deletion of USE IV results in low basal mRNA transcription throughout all stages of development tested. This basal level of transcription is approximately 20% of that of the wild-type construct at 20 h postfertilization. Moreover, when placed downstream of the simian virus 40 late promoter, this element is able to activate transcription at the late blastula stage of development (36). Thus, USE IV can act as an enhancer of gene activity and direct proper temporal gene activation. On the basis of in vivo competition assays as well as in vitro footprinting data, we were able to identify a *trans*-acting factor which specifically binds to this embryonic enhancer (36).

In this report, we present data on the purification and characterization of the USE IV enhancer-binding protein, referred to as the stage-specific activator protein 1 (SSAP-1). Purified SSAP-1 from early-blastula stage extracts has a molecular mass of 43 kDa. Nuclear extracts from the early-embryonic stage organisms, in which H1-β transcription is low, show low but detectable levels of SSAP-1 DNA-binding activity. Although SSAP-1 DNA-binding activity increases during development, this increase precedes the activation of the H1-β gene. The disappearance of the 43-kDa species and the appearance of a 90- to 100-kDa SSAP species, however, correlate with H1-β gene activation. Western blot (immunoblot) and Southwestern (DNA-protein) analysis confirm the presence of the 43-kDa protein, SSAP-1, in the higher-molecular-weight complex. This novel, temporally regulated modification coincides with enhancer activation and is likely to account for the activation of the H1-β gene during embryogenesis.

MATERIALS AND METHODS

Construction of H1-β–CAT fusion constructs. A *Hin*cII fragment from the *S. purpuratus* H1-β gene (35), containing the sequence from approximately −1200 to +7, was cloned into the blunt-ended *Hin*dIII site of pIBI-CAT. This construct is referred to as GC315. pIBI-CAT is a promoterless chloramphenicol acetyltransferase (CAT) vector prepared by subcloning the *Bam*HI-*Hin*dIII fragment of pSV2CAT (18) into the pIBI31 vector. GC332 was prepared by subcloning a *Hin*dIII-*Bam*HI fragment containing the CAT gene as well as 227 bases upstream of the H1-β start site into pIBI31. A 33-bp oligonucleotide corresponding to USE IV of *S. purpuratus* H1-β was prepared by annealing complementary oligonucleotides of sequence 5′-AAGTTTTAAATCTGAT TCTGTAACTGTAAGTTT-3′. The gel purified 33-mer was phosphorylated with T4 polynucleotide kinase, catenated by treatment with T4 DNA ligase (52), and cloned into vector M13mp7. A clone with three copies was digested with *Bam*HI to release the insert fragment and ligated with *Bam*HI-treated GC332, resulting in GC337, which contains six copies of the USE IV sequence downstream of the CAT gene.

Microinjection of the sea urchin one-cell zygotes. The procedure used to inject the *L. pictus* zygotes was essentially that of McMahon et al. (44) and Colin (8) and was followed exactly as described by Lai et al. (37). All DNA constructs were first linearized by digestion with *Eco*RV and injected at a concentration of 10 μg/ml along with 40 μg of carrier *S. purpuratus* genomic DNA that had also been digested with *Eco*RV (40 μg/ml).

CAT assays. Fifty injected embryos were collected at 20 h postfertilization (hatched blastula). The embryos were washed in 0.25 M Tris-HCl (pH 7.8) and resuspended in 100 μl of the same buffer. Following three consecutive freeze-thaw cycles (45), CAT enzyme activity was assayed as described by Gorman et al. (18). The percent acetylation was calculated by dividing the number of counts of acetylated chloramphenicol by the total counts, which were measured by scintillation counting the appropriate regions of the thin-layer chromatography plates. CAT activities relative to the average background level were measured. Authentic bacterial CAT enzyme (Pharmacia) was used as a positive control.

Preparation of nuclear extracts. Crude nuclear extracts were prepared essentially by the method of Morris et al. (46). *S. purpuratus* embryos were fertilized in the presence of 1 mM *para*-aminobenzoic acid to prevent hardening of the fertilization membrane and grown at 16°C. Aliquots were removed at 4, 8, 12, and 24 h postfertilization. All steps were done at 4°C. Embryos were washed twice in 0.55 M KCl and once with washing buffer (0.25 M sucrose, 0.01 M Tris-HCl [pH 7.5], 0.1 mM EDTA). Then, they were resuspended in 5 to 10 volumes of buffer I (0.32 M sucrose, 0.01 M Tris-HCl [pH 8.0], 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 1 mM spermidine, 1 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride) and homogenized with 10 to 15 strokes in a Dounce homogenizer (pestle B). Nuclei were pelleted by centrifugation (3,500 rpm for 10 min in a Sorval SS34 rotor). Nuclei were washed three to four times in buffer I and recentrifuged. After washing, the nuclear pellets were resuspended in buffer III (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 0.1 M KCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride). The chromatin was extracted by incubating the pellets with 0.4 M $(NH_4)_2SO_4$ for 30 min and centrifuged at 35,000 rpm for 1 h in a Beckman 60 Ti rotor. The supernatant was collected, and the protein was precipitated by the addition of 0.25 g of $(NH_4)_2SO_4$ per ml of volume. The solution was centrifuged, and the protein pellet was resuspended in a minimum of buffer III plus 20% glycerol and dialyzed against the same buffer. Protein concentrations were determined by using the Bio-Rad protein assay.

Purification of SSAP-1. Nuclear extracts were prepared from blastula stage embryos (12 h) as described above, except that protein pellets were resuspended in buffer IV (50 mM Tris-HCl [pH 7.9], 0.1 mM KCl, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 20% glycerol) and dialyzed until the conductivity reached 0.1 M KCl. The protein extract was diluted to 20 mg/ml, and between 200 and 300 mg of protein was applied to a Sephacryl S300 column equilibrated with buffer IV. Protein elution was monitored by A_{280}, and SSAP-1 activity was assayed by mobility shift assay analysis of the column fractions. Fractions from three columns were pooled and precipitated by 60% saturated ammonium sulfate and centrifuged at 35,000 × *g* for 15 min. The pellet was resuspended at 10 mg/ml in buffer III and applied to a 2-ml DNA affinity column. The affinity resin used was prepared by using two complementary oligonucleotides of sequence 5′-GATCGACAAGTTTTAAATCTGAT TCTG-3′ (3, 26). Poly(dI-dC) at 50 μg/ml and poly(dA-dT) at 5 μg/ml were used as nonspecific carrier DNAs by being incubated with the extract for 30 min at 4°C before being loaded onto the column. SSAP-1 activity was eluted at 0.6 M KCl, and the appropriate fractions were pooled and reapplied to a 0.5-ml DNA affinity column with the poly(dI-dC) at

100 μg/ml and the poly(dA-dT) at 10 μg/ml. This procedure was repeated again under the same conditions.

Generation of anti-SSAP antiserum. Immune serum was generated by immunizing two rabbits via intradermal injections. Ten micrograms of third-pass DNA affinity-purified protein was first coupled to an equal amount of keyhole limpet hemocyanin by treatment with glutaraldehyde (21) and then injected into two rabbits. Immune titers were monitored by Western blot analysis. After the initial titer began to fall (at approximately 10 weeks), the animals were boosted in exactly the same manner. Immune serum against recombinant bacterial SSAP was also generated by immunizing two rabbits and will be described in another publication (12).

DNase I footprinting and mobility shift assay. DNase I footprinting reactions (16) and mobility shift assays were carried out as previously described (14, 36). Typically 1 to 2 fmol of the DNA probe was used for both assays, and poly(dI-dC) was included as a nonspecific competitor. The probe used for the mobility shift assay was prepared by annealing the same two oligonucleotides used in preparing the affinity resin described above. The oligomer was labeled by filling in the 5′ overhang with [α-^{32}P]dGTP and Klenow DNA polymerase. For competition studies, 50 ng of competitor (approximately 1,800-fold molar excess) was added to the reaction mixture before the addition of the extract. Both the specific and the nonspecific competitor DNAs were synthesized oligomers of approximately 30 bp in length and were used unligated.

The reaction with anti-SSAP antiserum was conducted by first incubating 3 μl of either preimmune or immune serum with 10 μg of nuclear extract for 2 h on ice in binding buffer (36). The nonspecific competitor and the ^{32}P-labeled probe were then added, and the incubation was continued on ice for an additional 30 min.

SDS-PAGE and Western blots. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (33), and silver staining was done by the technique outlined by Ansorge (1). High-molecular-weight markers were obtained from Amersham. For Western blots, samples were first run in SDS-PAGE and then electrophoretically transferred onto nitrocellulose (55). Blots were washed three times in TBSN (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% [vol/vol] Nonidet P-40) and then incubated in blocking buffer (4% [wt/vol] bovine serum albumin in TBSN) for 90 min at room temperature. The blots were then washed three more times in TBSN and incubated with antiserum at a 1:6,400 dilution in blocking buffer for 1 h and then washed five times (for 5 min each time) in TBSN. In order to detect bound antibody, the blots were incubated with peroxidase-conjugated donkey anti-rabbit antibodies (Amersham) and visualized by the ECL-enhanced chemiluminescence reagents provided by Amersham.

Southwestern analysis. Samples (approximately 100 μg each) of crude nuclear extracts were run in SDS-PAGE and electrophoretically transferred onto nitrocellulose. The blots were washed twice for 15 min in denaturation buffer (20 mM HEPES-KOH [pH 7.9], 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 6 M guanidine-HCl). The transferred proteins were renatured by washing with denaturation buffer which was serially diluted by one-half with buffer D (20 mM HEPES-KOH [pH 7.9], 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, 300 mM KCl, 20% glycerol) for 10 min at 4°C. This procedure was repeated five times. The blots were then washed twice for 5 min each time with buffer D. The blots were blocked by being incubated with 5% nonfat dry milk dissolved in buffer D twice for 20 min each time and then washed in 0.25% nonfat dry milk in buffer D twice for 10 min each time. The blots were probed by using 1×10^6 to 5×10^6 cpm of radiolabeled DNA, 5 μg of denatured salmon sperm DNA, and 50 μg of poly(dI-dC) per ml overnight at 4°C with gentle agitation. The blots were washed with buffer D containing 0.25% nonfat dry milk five to six times for a total of 30 min and then autoradiographed.

The probe was prepared by phosphorylating the 27-bp oligonucleotide containing the nucleotide sequence corresponding to USE IV with polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP. The phosphorylated probe was then ligated to form catenates with an average of 15 to 20 copies of the oligomer.

RESULTS

USE IV enhancer activity. To determine whether USE IV is able to activate gene transcription from a homologous promoter in a position-independent manner, we generated a series of DNA constructs. GC315 was constructed by subcloning a 1,200-bp fragment containing the H1-β promoter region (from about −1200 to +7) upstream of the bacterial CAT gene. We then generated GC332 by removing USE IV and all upstream sequences (Fig. 1A). This construct contains only the TATA box and USEs 0 to III of the H1-β promoter. Six copies of a 33-bp double-stranded oligonucleotide corresponding to USE IV were cloned into GC332 downstream of the bacterial CAT gene, giving rise to GC337. These plasmids were first linearized and then microinjected into *L. pictus* one-cell zygotes. CAT enzyme activity was assayed at 20 h postfertilization, the time point corresponding to the peak of H1-β gene activity. The half-life of the CAT enzyme in a sea urchin embryo has been estimated to be about 40 min (15). Thus, the detection of CAT enzyme activity during a given stage is a reflection of the rate of transcription during that interval.

The deletion of USE IV and all upstream sequences (construct GC332) had an approximately fivefold reduction in CAT activity compared with that of the wild-type construct GC315 (Fig. 1B). This level of reduction was also observed by Lai et al. (37) with a construct which contained only a 30-bp internal deletion of USE IV. This suggests that USE IV is responsible, at least in part, for the embryonic activation of the H1-β gene. Microinjection of construct GC337, which contains six copies of USE IV, resulted in a 14.6-fold increase in CAT activity compared with that of the construct with a deleted USE IV. These measurements were taken in embryos at 20 h postfertilization, the time point corresponding roughly to the peak of late histone gene transcription (30, 32, 35). DNA quantitation by slot blot analysis confirmed that the constructs shown in Fig. 1 had replicated to a similar degree (Fig. 1C) and were saturating with respect to their abilities to direct CAT transcription (34). Thus, the differences among the activities of the CAT constructs were not due to the inability to replicate nor to loss of the injected DNA. Rather, this result demonstrates that USE IV is able to activate gene transcription in a position-independent manner from its homologous promoter and can, thus, function as an enhancer. USE IV is also able to activate CAT gene transcription from a minimal basal promoter containing only 106 bp of upstream sequences (TATA, USE 0, and USE I) as well as a TATA box alone (36, 51).

DNA-binding specificity of SSAP. We had previously demonstrated an activity in *L. pictus* whole-cell extracts that

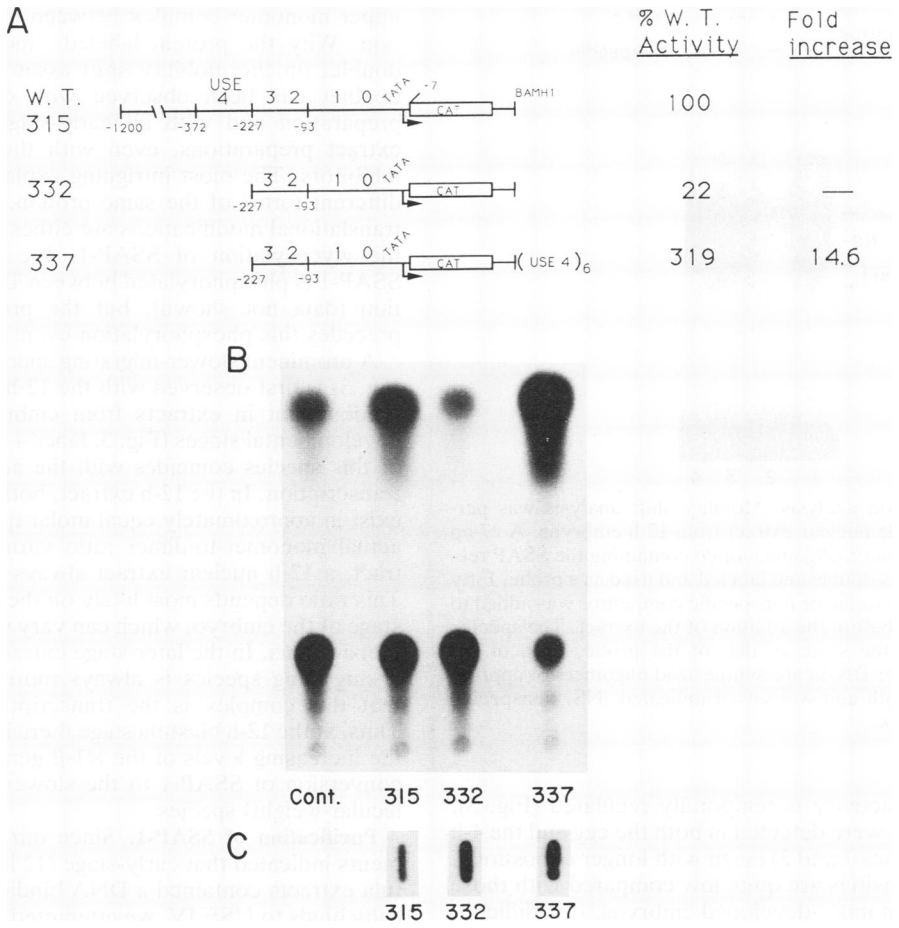

FIG. 1. Enhancer activity of the USE IV oligonucleotide. (A) Constructs used to test the enhancer activity of USE IV: pGC315, which contains approximately 1.2 kb of upstream H1-β sequences cloned 5′ of the bacterial CAT gene; pGC332, which is the same as pGC315, except that the sequences upstream of −227, including USE IV, were deleted; and pGC337, which is the same as construct pGC332, except that six copies of a 33-bp oligonucleotide corresponding to USE IV were cloned into the *Bam*HI site. The percentage of wild-type (W. T.) activity is based on the percentage of chloramphenicol that was acetylated. (B) Results of CAT assays of microinjected DNA constructs. Linearized DNA (2 pl of a 25-ng/μl solution) was introduced into fertilized one-cell *L. pictus* zygotes. Embryos were collected at 20 h postfertilization, and extract equivalent to 50 injected embryos was assayed for CAT enzymatic activity. The control (Cont.) lane contains a known quantity of authentic CAT enzyme. (C) DNA quantitation was determined by slot blot hybridization.

binds to the USE IV enhancer sequence (36). The specificities of the protein DNA complexes in *S. purpuratus* nuclear extracts were determined by using competition analysis and the mobility shift assay. Before addition of the ^{32}P-labeled probe, *S. purpuratus* nuclear extracts from 12-h blastula stage embryos were preincubated with either 50 ng of the same oligonucleotide as the probe or a nonspecific oligonucleotide of similar length (approximately 1,800-fold molar excess). This nuclear extract corresponds to a developmental stage that contains all of the specific DNA-protein complexes that will be discussed below. In the competition analysis, we observe three major complexes which compete only with the specific competitor (Fig. 2). The nonspecific complex, however, is inhibited by some but not all competitors. The reason for this is unclear. The results of experiments to be described below have led us to believe that the bottom two specific complexes arise from the SSAP monomer and the upper specific complex arises from a higher-molecular-weight species of SSAP that is likely to be a dimer. Although the complete composition of the higher-molecular-weight species is not known, it contains at least one 43-kDa SSAP polypeptide (see below). In addition to these three complexes, we frequently see specific complexes that migrate more slowly than the dimer.

Temporal activity of SSAP-1. In order to correlate SSAP-1 DNA-binding activity with the transcriptional activity of the H1-β gene, we prepared a series of nuclear extracts from embryos at various stages of development. To minimize any discrepancy in the nuclear extracts, we prepared all of the extracts from a single fertilization and removed aliquots of embryos at various points in development. Extracts were prepared from eggs (whole-cell extracts) and 4- and 8-h morula embryos, which correspond to the low basal level of H1 gene activity. Extracts were also prepared from 12- and 24-h blastula embryos corresponding to stages when H1-β gene transcription is increasing to its maximal level, which occurs at about 24 h (35). The increase in transcription during the blastula stage is a result of the activation of the enhancer element (36, 37) and, thus, thought to be regulated, at least in part, by SSAP-1.

The mobility shift experiments using extracts from embryos at various stages of development demonstrated that

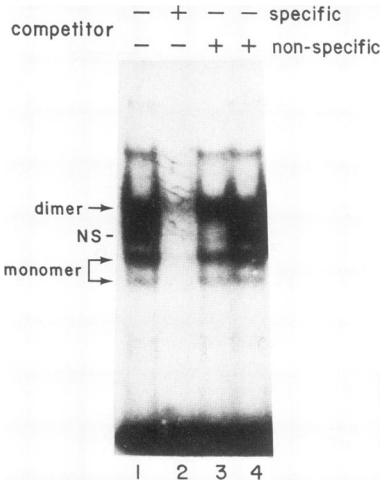

FIG. 2. Competition analysis. Mobility shift analysis was performed by using crude nuclear extract from 12-h embryos. A 27-bp double-stranded synthetic oligonucleotide containing the SSAP recognition sequence was double end labeled and used as a probe. Fifty nanograms of either specific or nonspecific competitor was added to the reaction mixture before the addition of the extract. The specific competitor DNA is the same as that of the probe. Both of the nonspecific competitor DNAs are synthesized oligomers of approximately 30 bp in length and were used unligated. NS, nonspecific protein-DNA complex.

the DNA-binding activity is temporally regulated (Fig. 3). Specific complexes were detected in both the egg and the 4-h extracts (Fig. 3, lanes 1 and 2) (seen with longer exposures), although their intensities are quite low compared with those of the extracts from more-developed embryos. It is difficult, however, to quantitate the SSAP activity in the whole-cell egg extract relative to the other nuclear extracts. High levels of activity were first detected with the 8-h extract (Fig. 3, lane 3) and with extracts from embryos of all subsequent stages tested. The two specific complexes labeled "monomer" are the same as those observed with the affinity-purified protein (Fig. 3, lane 6; also see below). There is a distinct transition from the lower band of the monomer to the

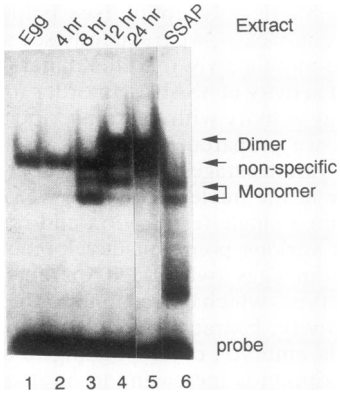

FIG. 3. Developmental profile of SSAP-1 and its DNA-binding activity. Mobility shift analysis was performed by using a crude whole-egg extract (10 μg) (lane 1), crude nuclear extracts (10 μg each) from 4-, 8-, 12-, and 24-h embryos (lanes 2 to 5, respectively), and a purified SSAP monomer (lane 6). Free probe as well as specific monomer and dimer complexes are indicated on the right.

upper monomer complex between 8 and 12 h postfertilization. Why the protein labeled "monomer" appears as a doublet on the mobility shift assay remains unclear. This doublet has been observed with every purified SSAP-1 preparation and with all early-stage (4 to 12 h) nuclear extract preparations, even with the addition of protease inhibitors. The most intriguing explanation is that they are different forms of the same protein. The most likely post-translational modifications are either the phosphorylation or the glycosylation of SSAP-1. We have determined that SSAP-1 is phosphorylated between 12 and 17 h postfertilization (data not shown), but the presence of the doublet precedes this phosphorylation event.

A prominent slower-migrating species, labeled "dimer" in Fig. 3, is first observed with the 12-h blastula extract and is predominant in extracts from embryos of all subsequent developmental stages (Fig. 3, lanes 4 and 5). The appearance of this species coincides with the activation of H1-β gene transcription. In the 12-h extract, both species exist and they exist in approximately equal molar quantities. Although the actual monomer-to-dimer ratio varies from extract to extract, a 12-h nuclear extract always contains both species. This ratio depends most likely on the precise developmental stage of the embryo, which can vary slightly among different preparations. In the later-stage extracts, however, the slower-migrating species is always more abundant, suggesting that this complex is the transcriptionally active species. Thus, at the 12-h blastula stage there is a correlation between the increasing levels of the H1-β gene transcription and the conversion of SSAP-1 to the slower-migrating (higher-molecular-weight) species.

Purification of SSAP-1. Since our initial binding experiments indicated that early-stage (12 h postfertilization) blastula extracts contained a DNA-binding activity that specifically binds to USE IV, we attempted to purify the enhancer-binding protein from such extracts (Table 1). The crude nuclear extract was first applied to a Sephacryl S300 molecular sieve chromatography column. Approximately 750 mg of the extract was applied (in two or three separate runs) to the column in a Tris buffer containing 0.15 M KCl so as to minimize protein-protein interactions (Fig. 4A). Every other fraction of the S300 column was analyzed by the mobility shift assay (Fig. 4B). The different SSAP monomer and dimer activities are separated by this gel filtration column, but the yields of the dimer are sometimes lower than expected. Fractions 31 to 46 contained an activity corresponding to the fastest-migrating DNA-binding complex (monomer) in the crude nuclear extract. Moreover, this complex is specific with regard to competition analysis (data not shown), and proteins from these fractions are fully able to protect the USE IV sequence from DNase digestion (see below).

The fractions which contained this specific faster-migrating form of the USE IV DNA-binding activities were pooled and precipitated with ammonium sulfate. The protein was resuspended at a concentration of 20 mg/ml, dialyzed against a 100 mM KCl-HEPES buffer (buffer III [see Materials and Methods]). This protein was applied to a 1-ml DNA affinity column in the presence of nonspecific competitor DNA. We were able to achieve a two- to threefold greater level of purification by using both poly(dI-dC) and poly(dA-dT) rather than using poly(dI-dC) alone. This is due most likely to the removal of many of the DNA-binding proteins which bind to AT-rich sequences. Fractions were again analyzed by the mobility shift assay. Specific DNA-binding activity was recovered in fractions with a high concentration of salt

TABLE 1. Purification of SSAP-1[a]

Procedure	Total protein (mg)	Total activity (f units[b])	Sp act (f units/mg)	Purification factor	Cumulative yield (%)
Nuclear extract	750	15,000[c]	20		100
Sephacryl S300	65	15,000	230	11.5	100
DNA affinity pass					
1	1.3	7,000	5,400	23.5	47
2	0.12	5,200	43,000	8.0	35
3	0.04	3,500	87,500[d]	2.0	23

[a] From 4×10^8 12-h blastula embryos.
[b] An f unit (footprint unit) is defined as the amount of protein needed to completely protect approximately 1 fmol of the probe.
[c] Since an accurate determination cannot be made, this value is an estimate, assuming 100% recovery at the Sephacryl S300 step.
[d] This number corresponds to approximately 11 ng/f unit.

(0.6 M KCl). The high-salt concentration eluate was pooled and reapplied two more times to smaller DNA affinity columns in the same manner (Fig. 4C). Although only SSAP monomer activity was pooled from the Sephacryl column, a significant amount of dimer activity is present in the more concentrated DNA affinity column eluate. This suggests that SSAP is able to dimerize with itself to form a homodimer.

Individual fractions eluted from the third affinity column were analyzed by SDS-gel electrophoresis and then silver stained, revealing two prominent species of 43 and 32 kDa and a minor species of 25 kDa (Fig. 4D). During many independent preparations, the USE IV DNA-binding activity always copurified with the 43-kDa protein throughout the purification process. The yield of the 32- and 25-kDa polypeptides was variable and likely caused by proteolysis. We refer to these polypeptides as SSAP-1. Typically, we purified 20 to 40 μg of SSAP from 750 mg of nuclear extract, and from this extract we estimate that SSAP-1 was purified approximately 4,000-fold (Table 1). The yield and fold purification are based on silver staining of the purified protein relative to standards and could overestimate the final yield of protein.

To determine whether purified SSAP-1 possesses the expected DNA-binding properties, column fractions generated during the purification process were assayed by DNase I footprint analysis. Three distinct footprints were detected by using crude nuclear extracts and the 155-bp probe (Fig. 5, lane 2), which contains sequences from −372 to −227 of the H1-β gene (35). Purified SSAP-1, however, protected only two of these sequences (Fig. 5, lanes 2 to 5). The first region, from −318 to −308, contained sequence GTTTTAAATCT, which corresponds to USE IV. The second protected region, from −257 to −247, contained sequence TTTATTAATTA. This additional protected region lies between USE III and USE IV, and the biological significance of this site is unknown. An interesting observation is that both sites are very AT rich and similar to the consensus sequence of the binding sites deduced for a large number of *Drosophila* homeodomain proteins (TCAATTAAAT) (23). The other footprint generated by the crude nuclear extract was due to an additional protein that did not bind to the affinity resin. Recently, we have discovered that the other highly conserved late H1 specific element, USE III (37), also binds SSAP-1 (51). Thus, the stage-specific enhancer within the H1-β gene is composed of three binding sites for a single protein, SSAP-1.

We were able to restore specific USE IV DNA-binding activity by eluting the 43-kDa polypeptide from an SDS-polyacrylamide gel, treating the eluted protein with guanidine hydrochloride, and then renaturing it by dialysis against buffer IV (Fig. 6). This SSAP-1 preparation contains few if any of the 32- and 25-kDa polypeptides. The retarded complex of the eluted 43-kDa protein (gel slice 2) was identical to that of the faster-migrating species of a doublet (the monomer) that is seen as a specific complex in crude nuclear extracts from early-stage embryos. Gel slices from other regions of the SDS-polyacrylamide gel contained no DNA-binding activity with the USE IV probe. We also utilized a UV cross-linking protocol to confirm the size of the SSAP polypeptide from crude nuclear extracts (12). Finally, we have isolated a cDNA encoding SSAP-1 which contains an open reading frame that predicts a polypeptide of 44 kDa, specifically binds to the USE IV sequence, transactivates target reporter genes, and has virtually the same amino acid composition as that of the gel-purified 43-kDa SSAP protein (13).

To determine whether the faster- and slower-migrating complexes are related to one another, we generated polyclonal immune serum from affinity-purified protein isolated from 12-h nuclear extracts. A modification of the mobility shift assay was used to determine whether the higher-molecular-weight complex that appears only later in development is related to purified SSAP-1. First, 3 μl of either preimmune or immune serum was incubated with 10 μg of nuclear extract for 2 h on ice. The radiolabeled probe and poly(dI-dC) were then added, and the reaction mixture was incubated on ice for 30 min before being subjected to PAGE. The antibodies which interact with SSAP-1 both inhibit SSAP binding and form an antibody–SSAP-1–DNA complex that migrates through the gel more slowly than the SSAP-1–DNA complex. The nonspecific complex remains unaffected by the immune serum and is, therefore, unrelated to SSAP-1 (Fig. 7). The three other complexes, however, are both inhibited and form an antibody–SSAP-1–DNA complex, demonstrating that the slower-migrating species is antigenically related to SSAP-1.

Dimerization of SSAP-1. The slower-migrating species appearing in the mobility shift assay beginning at 12-h postfertilization (Fig. 3) most likely represents either a homodimer of SSAP-1 or the interaction of SSAP-1 with another cellular protein(s). In order to determine the molecular weight of the slower-migrating species of SSAP, we subjected the 12-h nuclear extract to sedimentation through a glycerol gradient. The gradients were sedimented under low-salt concentration conditions to keep noncovalently associated complexes together. Marker proteins were either cosedimented with the nuclear extract or sedimented in a separate, parallel gradient. SSAP-1-binding activity was monitored by the mobility shift assay. The faster-migrating species in the mobility shift assay (the complexes labeled

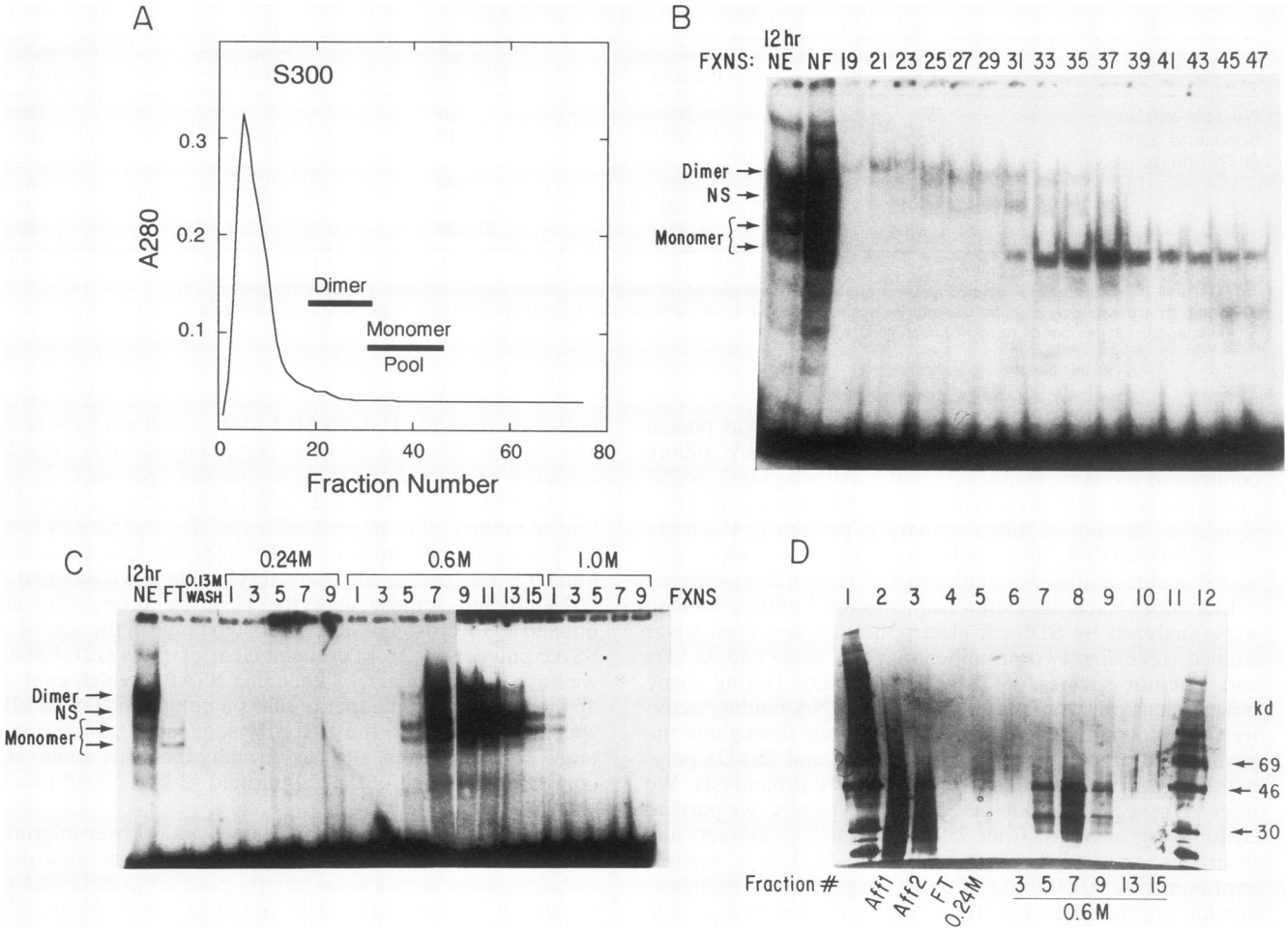

FIG. 4. Purification of SSAP activity. (A) Optical density profile of a crude 12-h blastula stage nuclear extract fractionated on an S300 Sephacryl column as described in Materials and Methods. The fractions containing SSAP monomer activity that were pooled and used in the DNA affinity column are indicated. (B) Mobility shift analysis of the Sephacryl S300 fractions (FXNS). Mobility shift assays were performed by using crude nuclear extract (NE) from 12-h early-blastula stage embryos and the indicated fractions from the Sephacryl S300 molecular sieve column. A 27-bp double-stranded synthetic oligonucleotide containing the nucleotide sequence corresponding to USE IV was used as a probe. Only the region containing DNA-binding activity is shown; however, all fractions were tested. NS, nonspecific protein-DNA complex. NF, nonfractionated extract (starting material). (C) Mobility shift analysis of the DNA affinity column fractions. Mobility shift analysis was performed by using crude nuclear extract from 12-h early-blastula stage embryos and the indicated fractions of the affinity column. The probe used was the same 27-bp oligonucleotide as in panel B. FT, flowthrough. (D) SDS-PAGE of affinity-purified SSAP. Lane 2, protein from a first-pass (Aff1) DNA affinity column eluted at 0.6 M KCl; lane 3, protein from a second-pass (Aff2) DNA affinity column eluted at 0.6 M KCl; lane 4, material from the flowthrough (FT) fraction; lane 5, protein eluted at 0.24 M KCl; lanes 6 to 11, protein fractions from a third-pass DNA affinity column eluted at 0.6 M KCl. Lanes 1 and 12, molecular weight markers whose sizes are indicated on the right.

"monomer") comigrated with ovalbumin at 3.5S (Fig. 8). Assuming a globular protein with average partial specific volume, this corresponds to a molecular mass of about 45 kDa. Since both complexes generated by the monomer form of SSAP-1 comigrate in the glycerol gradient, they probably differ by a posttranslational modification of the 43-kDa polypeptide SSAP-1 which does not alter its mobility through a glycerol gradient. The slower-migrating species in the mobility shift assay sedimented at a value of 5S, corresponding to a molecular mass of approximately 90 to 100 kDa (Fig. 8). These results demonstrate that both the faster- and the slower-migrating species are separable from one another. This is an important observation, demonstrating that the dimer is a stable species and not a result of the increase in SSAP-1 protein concentration during development.

We were next interested in independently confirming that the 43-kDa protein is a component of the 90- to 100-kDa slower-migrating species. We also attempted to obtain a more precise molecular weight of this species by chromatography on a Superose-12 fast protein liquid chromatography (FPLC) gel filtration column. A nuclear extract containing almost all of the SSAP activity in the 90- to 100-kDa higher-molecular-weight species (approximately 1 mg of total protein) was fractionated on the Superose-12 column, and portions (15 μl each) of each individual fraction were assayed by the mobility shift assay using a USE IV probe (Fig. 9A). Sequence-specific SSAP dimer activity, as defined by competition analysis (data not shown), was reproducibly eluted in fractions 34 to 36 between myoglobin and vitamin B12 size standards with an apparent molecular mass of approximately 2 to 3 kDa. The activities in fractions 23 to 26

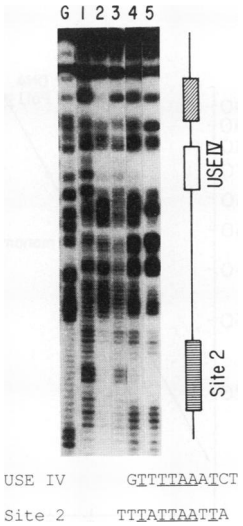

FIG. 5. DNase I footprint analysis of crude and purified SSAPs. A DNA fragment extending from −227 to −372 was used to assay DNase I protection on the coding strand. Lane G, Maxam-Gilbert sequencing reaction on the 155-bp fragment used as a marker; lane 1, DNase I digestion in the absence of added extract; lane 2, crude nuclear extract (55 μg) from 12-h blastula stage embryos; lane 3, fraction (7 μg) from the Sephacryl S300 column; lane 4, protein (10 footprint units) from the first-pass DNA affinity column eluted at 0.6 M KCl; lane 5, protein (10 footprint units) from the second-pass DNA affinity column eluted at 0.6 M KCl. A comparison of the SSAP recognition sequences is shown at the bottom. USE IV; the blastula stage embryonic enhancer; Site 2, another SSAP-binding site at positions −252 to −264 between USE III and USE IV.

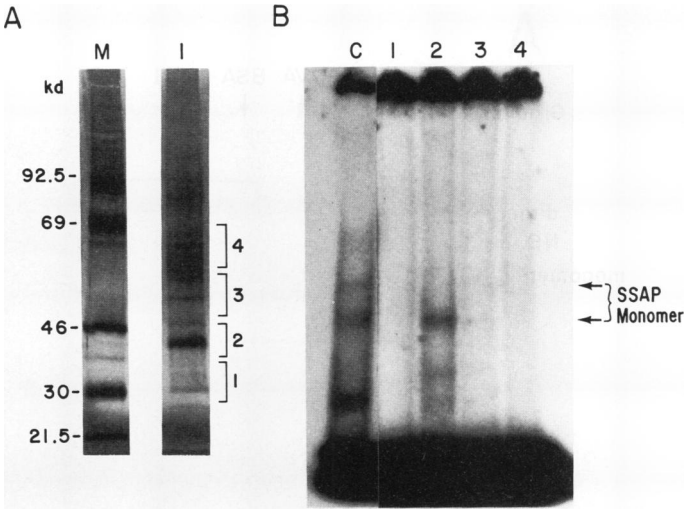

FIG. 6. Identification of the polypeptide containing USE IV DNA-binding activity. (A) Analysis of partially purified SSAP-1 by SDS-PAGE and silver staining. The protein was eluted at 0.6 M KCl from a second-pass DNA affinity column [by using poly(dI-dC) and not poly(dA-dT) as a nonspecific competitor]. Approximately 60 footprint units was loaded on the gel in a lane adjacent to the one used for silver staining. The corresponding gel slices (indicated by numbers on the right) were cut out, and the protein was eluted and tested for DNA-binding activity by the mobility shift assay. Lane M, molecular weight markers. (B) Mobility shift assay using the protein renatured from individual gel slices. Lane C, a control using protein (0.8 footprint unit) from a second-pass DNA affinity column eluted at 0.6 M KCl; lanes 1 to 4, protein eluted from the gel slices indicated in panel A.

are nonspecific. The 90- to 100-kDa SSAP species must interact with the Superose-12 resin, resulting in a delayed elution from the column under these conditions. Using a different nuclear extract, we observed that SSAP monomer is similarly delayed in its elution from the Superose-12 column (data not shown). One-half (250 μl) of each fraction of the column shown in Fig. 9A was trichloroacetic acid precipitated and tested for the presence of the 43-kDa SSAP protein by Western blot analysis using antiserum generated against recombinant bacterial SSAP (13). The elution of the 43-kDa SSAP protein perfectly coincides with the slower-migrating (90- to 100-kDa) SSAP species (Fig. 9B). The SSAP activity first appears in fraction 34, peaks in fraction 35, and trails off until fraction 40. We conclude that the 90- to 100-kDa SSAP species includes at least one 43-kDa SSAP monomer polypeptide.

The elution of the SSAP activity at an apparent molecular mass of 2 to 3 kDa suggested that Superose-12 column chromatography could be a very effective purification step. We, therefore, analyzed the polypeptide profiles of fractions 33 (no SSAP activity), 35-36 (the peak of SSAP activity), and 39 by SDS-PAGE and silver staining (Fig. 9C). Several faint polypeptides ranging in size from 60 to 30 kDa, including a small amount of a 43-kDa protein, are visible in fraction 33 (longer exposures of the Western blot detect small amounts of SSAP in fraction 33). Fraction 35-36 (75-μl portions of the two fractions were pooled and coprecipitated) contains a prominent 43-kDa protein that comigrates with bacterial SSAP. Two prominent polypeptides of about 35 and 37 kDa, in addition to several other faint protein species, are also seen. If the slower-migrating SSAP species is a heterodimer, then the only candidates for proteins that interact with SSAP-1 are the 35- and 37-kDa polypeptides. These polypeptides are not proteolytic fragments of SSAP, since they are not detected by Western blot analysis. Fraction 39 contains small amounts of the 43-kDa SSAP polypeptide, as expected on the basis of the Western blot, but not enough

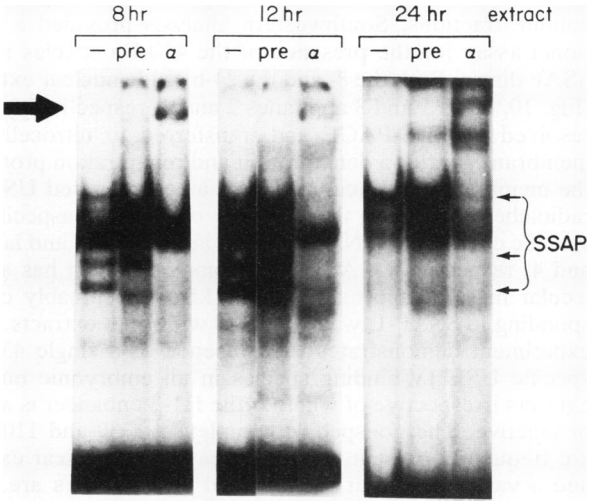

FIG. 7. Specificity of the anti-SSAP antibody. Crude nuclear extracts (10 μg each) from 8-, 12-, and 24-h embryos were incubated with either preimmune serum or anti-SSAP antiserum, and then a mobility shift assay was performed. The antibody-SSAP-DNA complex is indicated by the arrow on the left.

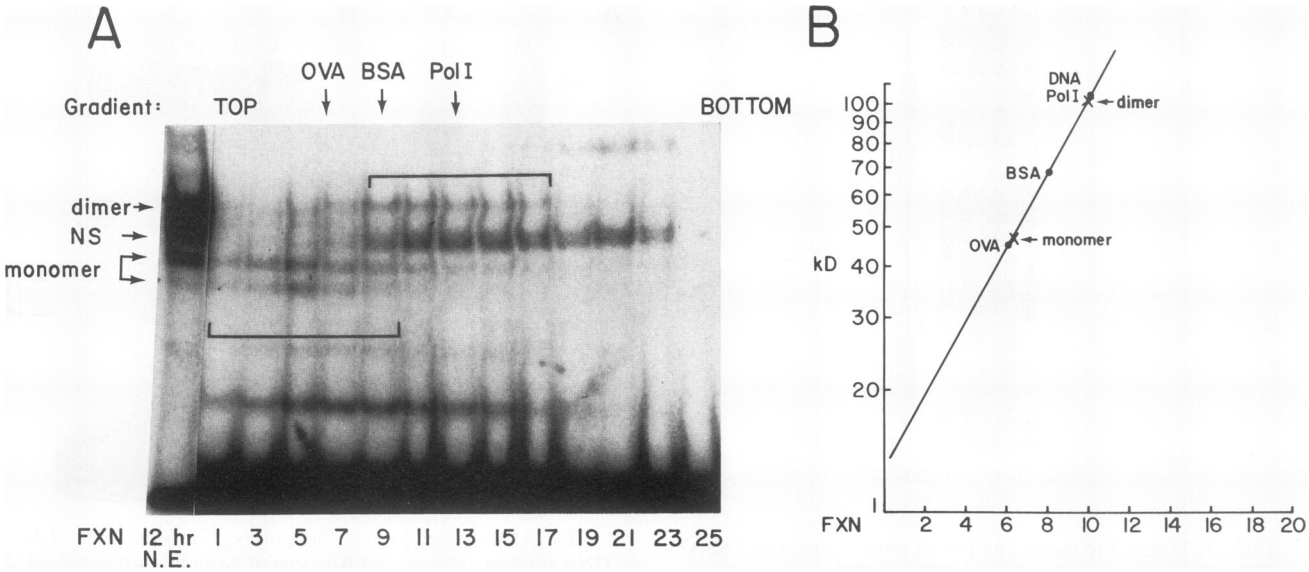

FIG. 8. Glycerol gradient sedimentation. (A) A 12-h crude nuclear extract (N.E.) (1 mg) was sedimented in a 10-to-30% linear glycerol gradient (49). Aliquots (5 μl each) of gradient fractions (FXN) were assayed in the standard mobility shift assay using a 27-bp oligonucleotide probe containing the SSAP recognition sequence. The sedimentation positions of the marker proteins are indicated at the top. NS, nonspecific protein-DNA complex. Brackets define the fractions with monomer and dimer SSAP activity. (B) The peak fraction to which each complex sedimented was determined by the peak DNA-binding activity as analyzed by the mobility shift assay (see panel A). The marker proteins were ovalbumin (OVA), bovine serum albumin (BSA), and *Escherichia coli* DNA polymerase I (Pol I).

protein is present in this fraction to see whether the 35- and 37-kDa proteins are present in quantities equimolar to the quantity of the SSAP-1 protein. Further work is clearly necessary to elucidate the polypeptide composition of the slower-migrating species of SSAP. Nevertheless, SSAP-1 seems to undergo a temporally regulated protein-protein interaction. Both the 8- and the 24-h nuclear extracts generate the same DNase I footprinting pattern (37; also data not shown), suggesting that the dimer is not interacting with any additional sequences.

In addition to immunoblot analysis of the Superose-12 column fractions, Southwestern analysis provided a functional assay for the presence of the 43-kDa species in the SSAP dimer. Both the 8- and the 24-h crude nuclear extracts (Fig. 10, lanes 1 and 3 and lanes 2 and 4, respectively) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After a denaturation and renaturation protocol, the membrane was incubated with a concatenated USE IV radiolabeled probe in the presence of either nonspecific or specific competitor DNAs (Fig. 10, lanes 1 and 2 and lanes 3 and 4, respectively). A specific complex, which has a molecular mass of approximately 43 kDa, presumably corresponding to SSAP-1, was observed with both extracts. This experiment demonstrates the presence of a single 43-kDa specific USE IV-binding species in all embryonic nuclear extracts irrespective of whether the H1-β enhancer is active or inactive. The nonspecific complexes at 30 and 110 kDa are frequently present when the sea urchin nuclear extract and a variety of other radiolabeled DNA probes are used (4, 14a). These experiments clearly demonstrate that the 43-kDa SSAP-1 polypeptide is present throughout embryogenesis and, more importantly, is a constituent of the SSAP dimer which is formed during the blastula stage of development.

DISCUSSION

We are interested in studying the mechanisms regulating temporal gene expression during embryogenesis. Many of the genes which regulate temporal gene activity in *Drosophila melanogaster* encode transcription factors, and in most cases the mere presence or absence of these factors or their ability to bind DNA seems to dictate the transcriptional activity of the target gene. This appears to be the case for the alcohol dehydrogenase (*Adh*) (22) and the Ultrabithorax (*Ubx*) promoters (2). In the case of the *Ubx* promoter, multiple sequence-specific transcription factors which bind to essential *cis*-activating elements were detected, and the activities of some of these factors vary during embryogenesis. With respect to the distal *Adh* promoter, however, the presence of a single factor (*Adf-1*) seems to correlate with transcriptional activity. The sea urchin early histone H3 gene seems to resemble more closely the *Ubx* promoter (14). Proteins bound at four different and distinct sites were detected only at stages when the H3 gene is transcriptionally active. This mechanism differs from the mechanism used by the late H1 gene, in which a single *cis* element, USE IV, plays a major role in determining proper temporal gene activation.

In this report, we demonstrate that the *cis* element USE IV is capable of activating the H1-β gene in a position-independent manner and, thus, functions mechanistically as an embryonic enhancer. Previously, we had demonstrated that the *cis* element USE IV is able to activate CAT transcription from a heterologous promoter (36). In this case, the simian virus 40 early promoter was used and one or three copies of the 33-bp oligonucleotide corresponding to USE IV were cloned downstream of the CAT gene. The most striking result was that gene transcription was activated only during the blastula stage of development (be-

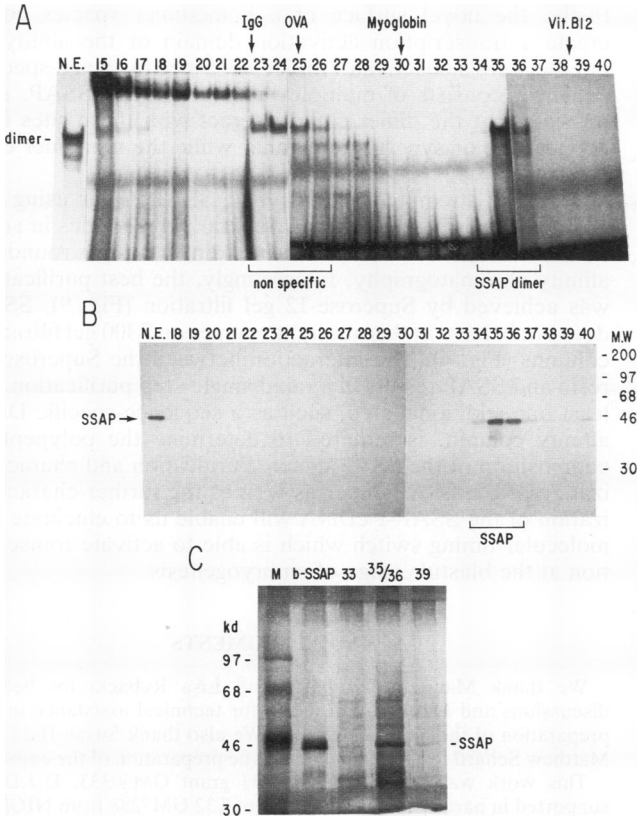

FIG. 9. Analysis of SSAP dimer activity by Superose-12 FPLC column chromatography. A 1-mg portion of nuclear extract (in 50 μl) was fractionated on a Superose-12 FPLC gel filtration column, and 500-μl fractions were collected. (A) Aliquots (15 μl each) of column fractions as well as the starting unfractionated nuclear extract (N.E.) were assayed in the standard mobility shift assay using a 27-bp oligonucleotide probe containing the SSAP recognition sequence. The elution positions of molecular size standards (Bio-Rad) run on the same column under the same conditions are shown. Competition analysis of selected fractions indicated that only fractions 34 to 36 contained specific DNA-binding activity. IgG, immunoglobulin G; OVA, ovalbumin; Vit. B12, vitamin B12. (B) Aliquots (250 μl each) of column fractions were trichloroacetic acid precipitated and used for Western blot analysis as described in Materials and Methods. Antiserum against recombinant bacterial SSAP was used at a dilution of 1:6,400. Molecular weights (MW) in thousands are shown on the right. (C) An aliquot (150 μl) of fraction 33, pooled aliquots (75 μl each) of fractions 35 and 36, and an aliquot of fraction 39 (150 μl) were trichloroacetic acid precipitated and analyzed by SDS-PAGE followed by silver staining. Included in the gel are molecular weight standards (lane M) and authentic recombinant bacterial SSAP (b-SSAP) (13).

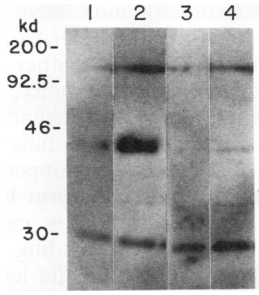

FIG. 10. Southwestern analysis of crude nuclear extract containing either an SSAP monomer or an SSAP dimer. Crude nuclear extracts from 8-h (monomer) (lanes 1 and 3) and 24-h (dimer) (lanes 2 and 4) embryos were resolved on SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods. After a denaturation and renaturation protocol, the membranes were incubated with a ligated radiolabeled oligomer USE IV probe in the presence of either specific (lanes 3 and 4) or nonspecific (lanes 1 and 2) competitor DNA. The competitor DNAs were prepared in the same fashion as the probe, and a 250-fold molar excess was used.

tween 12 and 18 h postfertilization). Thus, USE IV can function as an enhancer not only on its own homologous promoter but also on a heterologous promoter, implying that the USE IV-SSAP complex has the ability to interact with the transcriptional machinery of other genes. Thus, USE IV seems to be a general enhancer element and may activate many genes in a stage-specific manner during embryogenesis.

Utilizing both DNase I footprinting and mobility shift assays, we have identified a 43-kDa protein that interacts with this enhancer. We refer to this protein as SSAP-1. We employed several methods including renaturation of gel-purified protein, UV cross-linking, and Southwestern analysis to demonstrate that the 43-kDa polypeptide contains SSAP-1 activity. Using the method of Calzone et al. (5), we estimate that there are approximately 2,000 molecules of SSAP-1 per nucleus at the blastula stage of development. The interaction of SSAP-1 with USE IV is highly specific, with an affinity preference (K_r) for its target sequence of 6×10^4. In addition, purified SSAP-1 binds to sequence AGTTTTAAAT in USE IV with a K_d of 4.9×10^{10} (13), and we hypothesize that it is necessary to direct the activation of the H1-β gene above basal levels. Both of the *S. purpuratus* late H1 genes (H1-β and H1-γ) as well as the *L. pictus* H1-γ gene contain the USE IV sequence (31, 35). Purified SSAP-1 from *S. purpuratus* is also able to protect a similar sequence, GATTTTAAAG, in the USE IV region of the *L. pictus* H1-γ gene (data not shown). Thus, SSAP-1 is likely to be responsible for the coordinate activation of the embryonic gene transcription of both late H1 genes. The H1-β histone gene is not only expressed during embryonic development but is also expressed in somatic tissues (35). However, we have not yet tested for the presence of SSAP-1 in adult-organism tissues.

We have demonstrated that SSAP-1 undergoes a temporally regulated modification, as monitored by the mobility shift assay. From the early blastula stage (12 h) through successive stages of development, SSAP-1 is present in a slower-migrating (higher-molecular-weight) species. Our evidence for this is based on immunological conservation of epitopes on the monomer and dimer, glycerol gradient sedimentation, and Western blotting as well as Southwestern analysis of late-stage extracts. The slower-migrating species maintains the same DNA sequence-binding specificity as that of the faster-migrating species and, on the basis of its sedimentation through a glycerol gradient, has an apparent molecular mass of 90 to 100 kDa. The 43-kDa polypeptide SSAP-1 is clearly a component of this slower-migrating species, and it is this higher-molecular-weight complex of SSAP that appears to be generated by a temporally regulated protein-protein interaction.

Many DNA-binding proteins consist of homo- or heterodimers that have different DNA-binding or activating potentials. The leucine zipper DNA-binding–dimerization motif comprises the Jun/Fos (for reviews, see references 10

and 20), ATF/CREBP (19), and C/EBP (56) families. In most cases, these proteins can bind DNA only as dimers, but as either homo- or heterodimers. Another DNA-binding–dimerization motif is the helix-loop-helix, which was initially described for the immunoglobin enhancers E12 and E47 (47). Other members of the family include MyoD, Myf 5, and myogenin, and like the leucine zipper proteins, the helix-loop-helix family proteins can form both homo- and heterodimers. Heterodimer formation can increase both the number and the diversity of binding sites as well as the DNA-binding affinity. For both the leucine zipper and the helix-loop-helix families, there are examples of heterodimeric proteins that bind DNA significantly tighter than the respective homodimeric complexes. The Jun/Fos heterodimer, for example, binds to the AP-1 binding site with a significantly higher affinity than the Jun/Jun homodimer (20). The Fos/Fos homodimer is unable to bind to the AP-1 site. Similar results for the E12 and E47 enhancer proteins have been described (48). This is apparently not the case with SSAP. Both the SSAP monomer and the SSAP dimer are able to bind to USE IV with apparently equal affinities (51a). Since the early- and late-stage blastula extracts each give identical DNase I footprints, and given that USE IV does not contain a palindromic sequence, it seems doubtful that the second component of the SSAP-1 dimer is making contact with the DNA helix. If the complex is a homodimer, then two potential DNA-binding sites exist. The major histocompatibility complex class II regulatory factor (RFX) binds as a homodimer and, perhaps, forms a DNA loop by cross-linking two X-box elements found upstream of the major histocompatibility complex class II gene (50). This mechanism may be responsible for the transcriptional activation of the class II genes.

By 8 h postfertilization, SSAP-1 is relatively abundant, yet found only in the monomeric form. It is only after the 12-h stage of development that SSAP-1 begins to dimerize. The two most likely mechanisms for this are (i) that a second and distinct subunit (SSAP-2) of the SSAP heterodimer is available for interaction with SSAP-1 only in the late blastula stage of development and (ii) that one or both of these proteins require posttranslational modification to interact. The modification could be required to allow SSAP-1 homodimers or heterodimers to form. Our preliminary results suggest that SSAP-1 is phosphorylated between 12 and 17 h postfertilization (data not shown). Interestingly, this corresponds to both the presence of the dimer and the transcriptional activation of the H1-β gene. The regulation of the dimerization of SSAP-1 may be similar to that of the cyclic AMP response element-binding protein (CREBP) (59). CREBP undergoes a phosphorylation-dependent dimerization. The dimer-to-monomer ratio is increased by phosphorylation with protein kinase C and dramatically decreased upon phosphatase treatment. The dimeric CREBP complex, however, is a homodimer and, therefore, is able to bind with a greater affinity to its palindromic recognition sequence.

If SSAP is a heterodimer, a second subunit (SSAP-2) could function as a coactivator contributing a transcription activation domain to the DNA-binding domain of SSAP-1. In the early stages of development (morula and early blastula), SSAP-1 monomer is able to bind to USE IV but unable or only poorly able to interact with the transcriptional machinery, resulting in a low basal level of transcription. During the blastula stage of development, SSAP-1 then interacts with another nuclear protein(s), SSAP-2, and the resulting heterodimer is now able to interact with TFIID or another component of the basal transcriptional machinery. Alternatively, the novel surface of a homodimer species could create a transcription activation domain or the ability to interact with a coactivator molecule. Since the stage-specific enhancer consists of multiple binding sites for SSAP, it is possible that the dimer could interact with these sites in a cooperative or synergistic manner while the monomer cannot.

We have attempted to purify the SSAP dimer using the protocol described in Table 1. Several polypeptides in addition to the 43-kDa SSAP protein remain after three rounds of affinity chromatography. Interestingly, the best purification was achieved by Superose-12 gel filtration (Fig. 9). SSAP does not behave in this aberrant manner on S300 gel filtration columns (Fig. 4). The interaction between the Superose-12 resin and SSAP results in a rapid single-step purification. At least one additional step, such as a sequence-specific DNA affinity column, is required to determine the polypeptide composition of the SSAP dimer. Purification and characterization of the SSAP dimer as well as the further characterization of the SSAP-1 cDNA will enable us to elucidate the molecular timing switch which is able to activate transcription at the blastula stage of embryogenesis.

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