

Alteration by Heat Shock and Immunological Characterization of *Drosophila* Small Nuclear Ribonucleoproteins

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Abstract. Sera from human patients with systemic lupus erythematosus (SLE) have been shown to react with snRNP particles of both mammals and *Drosophila* (Mount, S. M. and J. A. Steitz. 1981. *Nucleic Acids Res.* 9:6351-6368). We have utilized fully characterized monospecific sera and specifically purified antibodies to carry out indirect immunofluorescence experiments with frozen sections of *Drosophila* embryos. Embryos subjected to severe heat shock before sectioning showed reduced binding of anti-Sm sera. Anti-nRNP sera reacted identically with antigens of heat shocked and non-heat-shocked sections. The reduction in anti-Sm fluorescence was restored by a brief salt wash. These results imply a non-covalent alteration in the conformation of Sm antigens with the administration of heat shock that can revert with exposure to salt. *Drosophila* antigens have been

compared to mammalian standards, showing partial identity with bovine spleen extract (BSE) antigens when reacted with anti-Sm sera. The antigenic relatedness between affinity-purified heat-shocked and non-heat-shocked *Drosophila* antigens and their mammalian homologues was examined by quantitative ELISA methodology. In all cases, the *Drosophila* antigens from heat-shocked and non-heat-shocked embryos were identical. We theorize that the heat shock-induced alteration of Sm antigen reverts during extraction. Because the snRNP antigens have been shown to be involved in splicing, and because splicing is inhibited during heat shock (Yost, H. J., and S. Lindquist. 1986. *Cell.* 45:185-193), our results provide information on the nature and stability of a change in these antigens which may be a central element in control of the heat shock response.

ANTIBODIES to either of two soluble nuclear antigens designated Sm and nRNP (Tan and Kunkel, 1966; Mattioli and Reichlin, 1971) are found in 46% of patients with systemic lupus erythematosus (SLE)¹ (Reichlin, 1987). These antigens have been implicated in RNA splicing and have become the focus of much research (Lerner and Steitz, 1979; Rogers and Wall, 1980). The antigens are most commonly referred to as small nuclear ribonucleoprotein particles (snRNPs) and consist of the U series of small nuclear RNAs complexed with proteins. The two major classes of snRNP antibodies are (a) anti-nRNP, which recognizes proteins unique to the U1 snRNP, and (b) anti-Sm, which reacts with proteins of the U1, U2, U4, U5, and U6 particles (Lerner and Steitz, 1979; Hinterberger et al., 1983; Kinlaw et al., 1983). Thus, the U1 particle contains antigens that are recognized by both major classes of snRNP antibodies.

We have used well-characterized sera from SLE patients to investigate the homologous particles in *Drosophila*. Because of the relationship between heat shock and reduced

splicing activity (Yost and Lindquist, 1986), we have used purified antibodies from these sera to investigate the snRNP particles of *Drosophila*.

Heat shock disrupts many cellular processes (for review, see Schlesinger et al., 1982), including RNA splicing (Yost and Lindquist, 1986). Yost and Lindquist (1986) demonstrated an inhibition of mRNA splicing during severe heat shock treatments (38°C, 15 min) although transcription rates appeared unaltered. A mild heat shock (33°C, 15 min) administered before the more severe treatment (38°C, 15 min) permitted splicing to continue during the restrictive treatment. However, the molecular mechanism involved in the effect of heat shock on processing of mRNA precursors is still unclear.

The results of the experiments reported here suggest a heat shock-induced alteration in *Drosophila* snRNP antigens, as assessed by indirect immunofluorescence in sections of heat-shocked and non-heat-shocked *Drosophila* embryos. A dramatic loss of fluorescence occurs in sections of heat-shocked embryos when SLE sera of anti-Sm specificity are used. Fluorescence is restored by a brief incubation of the heat-shocked sections in various concentrations of salt, indicating a noncovalent alteration may be responsible for the loss of

1. *Abbreviations used in this paper:* ACB, affinity column buffer; BSE, bovine spleen extract; CIE, counterimmunoelectrophoresis; CTE, calf thymus extract; hsp, heat shock proteins; PBST, PBS with 5% Tween-20; SLE, systemic lupus erythematosus.

the fluorescent signal with heat shock treatments. Immunochemical assays indicated no difference between the Sm antigens of extracts prepared from heat-shocked and non-heat-shocked 0–24-h *Drosophila* embryos, emphasizing the labile and reversible nature of the alteration. We also present comparative immunochemical data on the evolutionary relatedness of snRNP antigens in mammals and dipterans.

Materials and Methods

Immunoprecipitation

For each sample, 30 μ l of protein A-Sepharose (Boehringer-Mannheim Biochemicals, Indianapolis, IN) beads were incubated with 15 μ l of SLE sera or normal human serum in 500 μ l of immunoprecipitation buffer (10 mM Tris HCl; 0.5 M NaCl; 0.1% NP-40; pH 8.0) for 1 h at 4°C. They were extensively washed in NET-2 buffer (50 mM Tris HCl; 150 mM NaCl; 0.05% NP-40; pH 7.4) (McNeilage and Whittingham, 1984). An extract of *Drosophila* embryos was prepared from 1 g of heat-shocked or non-heat-shocked 0–24-h embryos by sonicating them in 6.65 ml of NET-2 buffer and 5% vanadyl ribonuclease inhibitor. The slurry was centrifuged at 10,000 rpm for 30 min at 4°C and the supernatant removed. 1 ml of extract was incubated with the coupled protein A-Sepharose beads for 1 h at 4°C. The beads were extensively washed in NET-2 buffer. The RNA was phenol-extracted, separated on a 10% polyacrylamide/7 M urea gel, and visualized with silver staining as described by McNeilage and Whittingham (1984).

The protein components of snRNPs were isolated by immunoprecipitation. The above procedure was followed with the addition of 0.004% PSMF during the extraction procedure. After incubation of the beads with the extract, the beads were washed five times with NET-2 buffer. In some cases, 10 μ g of RNase A (10 mg/ml in 10 mM sodium acetate, pH 5.5) was added along with 25 μ l of PBS and incubated at 37°C for 15–20 min. In other cases, the RNase was omitted and only 25 μ l of PBS was added. An equal volume of two times the sample buffer (Laemmli, 1970) was added to the beads and the mixture boiled for 10 min. The beads were centrifuged for 5 min to separate the beads from the buffer. The supernatant was loaded directly onto 10% or 15% SDS-polyacrylamide gels and electrophoresed for 40 min at a constant 200 V. The proteins were visualized by Coomassie blue staining.

Indirect Immunofluorescence

Drosophila melanogaster, Canton S strain, were raised at room temperature. 0–24-h embryos were collected and either embedded immediately in plastic medium for frozen sectioning (OCT; Tissue Tek II, Miles Laboratories Inc., Naperville, IL) or subjected to one of three treatments before preparation for sectioning. The embryos were either (a) exposed to heat shock at 33°C for 15 min; (b) exposed to heat shock at 33°C for 15 min and allowed to recover for 2 h at room temperature and exposed to heat shock at 37°C for 15 min; or (c) exposed to heat shock at 37°C for 1 h and then embedded. The frozen embryos were sectioned (8–10 μ m) with a cryostat (Ames Miles Laboratories Inc., Naperville, IL). The sections were acetone-fixed for 1 min and allowed to air dry. Other methods of fixation, while they resulted in improved morphological preservation, reduced the immunofluorescent signal.

Previously characterized sera of nRNP and Sm specificities from SLE patients were diluted in PBS, as was the fluorescein conjugated anti-human IgG. Anti-nRNP sera formed a single line in Ouchterlony experiments with crude calf thymus extracts (CTE) and bound strongly to nRNP-coated plates, but not to Sm-coated plates in ELISA experiments (Reichlin, 1987). Anti-Sm sera had a single line in Ouchterlony experiments with CTE and bound equally to nRNP and Sm-coated plates and were more than 60% anti-Sm as revealed by quantitative inhibition of binding to nRNP coated plates by affinity-purified Sm (Reichlin, 1987).

Sections were incubated for 20 min with dilutions of SLE sera or control (anti-DNA or normal human serum) at room temperature. They were washed three times in PBS for 2 min each. The fluorescein conjugated anti-human IgG (1:75 dilution) was then applied to the section and incubated 20 min at room temperature. The sections were again washed three times in PBS for 2 min each. A drop of mounting buffer (90% glycerol in PBS) was placed on each section and a glass coverslip applied.

SLE sera were preincubated with 200 μ g/ml of bovine nRNP or bovine Sm for 1 h at room temperature or overnight at 4°C and then used in the indirect immunofluorescence assay as described above.

To test the effect of varying salt concentrations, the frozen sections were

incubated for 20 min at room temperature in either ddH₂O or phosphate buffer (0.02 M phosphate) with 0.15, 0.5, or 1 M NaCl. After washing three times in PBS for 2 min each, the sections were processed for indirect immunofluorescence as described above. To stage the embryos for developmental age, visible light microscopy (200–400 \times) was used. Stages were determined by comparison to staged sections (Campos-Ortega and Hartenstein, 1985).

Preparation of Crude *Drosophila* Embryo Extracts

Drosophila 0–24-h embryos were collected and were either frozen immediately in liquid nitrogen or heat shocked at 37°C for 60 min and then frozen. The frozen embryos were homogenized with a polytron homogenizer (Brinkmann Instruments Co., Westbury, NY) in PBS and then sonicated. The resulting slurry was centrifuged at 18,000 rpm for 20 min at 4°C. The supernatant was removed and filtered through filter paper (No. 1; Whatman Inc., Clifton, NJ). The protein concentration was determined by protein assay (Bio-Rad Laboratories, Richmond, CA). The extracts were kept on ice during the procedure, and they were used immediately.

Affinity Purification

Details of the following procedures have been published (Reichlin, 1987). IgG was isolated from previously typed anti-nRNP and anti-Sm sera on DE52 columns equilibrated with 0.02 M K₂PO₄ buffer. Serum was dialyzed against this buffer and then passed over such columns. The effluent was pure IgG as judged by immunoelectrophoresis with anti-whole human serum. Yields were 5–15 mg IgG/ml of serum. Sera selected had the immunochemical specificity described in a previous section.

IgG was concentrated to 10 mg/ml in coupling buffer (Reichlin, 1987) and conjugated to cyanogen bromide activated sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). Proportions were 10.0 mg IgG/g wet weight gel and coupling efficiency was >85% as judged by residual IgG in solution after the coupling reaction.

These IgG-coupled resins with anti-nRNP and anti-Sm specificity were then poured into columns for use. Extracts of fresh calf thymus tissue were prepared by homogenization in PBS (100 g/200 ml) in a blender (Waring Products, New Hartford, CT) for 5 min and filtration through cheese cloth, followed by centrifugation in a refrigerated centrifuge (Sorvall Instruments Div., DuPont Co., Newton, CT) at 4°C for 30 min at 18,000 rpm. The supernatant was decanted and dialyzed against 10 vol affinity column buffer (ACB; 0.02 mM Tris HCl, 0.5 M NaCl, pH 7.2) overnight at 4°C. The dialyzed extract was then passed over the anti-nRNP column until the column was saturated, as indicated by the appearance of nRNP antigen in the effluent. The column was then washed with ACB until the effluent OD was <0.01 at 260 nm. The nRNP was then eluted with 3.0 M MgCl₂ and immediately dialyzed against PBS. The 260:280 ratio of this material varied between 1.5 and 1.6 and precipitated with anti-nRNP serum.

The effluent was repeatedly passed over such anti-nRNP columns until it was depleted of nRNP as indicated by its inability to block the nRNP-anti-nRNP reaction in ELISA. Such RNP-depleted extract was then passed over anti-Sm columns until the effluent failed to precipitate in Ouchterlony experiments with anti-Sm serum. The column was then washed until the effluent had an OD of 0.01 or less at 260 nm, and was then eluted with 3.0 M MgCl₂ and treated as the nRNP above. Protein was determined by both a 260:280 nomogram and dye reagent (Bio-Rad Laboratories) with BSA standard. These assays agreed within 20%.

Drosophila crude extracts were applied to the columns, rinsed with ACB, and the antigen eluted with 3 M MgCl₂ or 1 M acetic acid. The fractions were monitored by their absorbance at 280 nm and the high readings pooled. The pooled fractions were dialyzed to normal Tris buffer (0.02 M Tris HCl; 0.015 M NaCl; pH 7.2) and concentrated using a YM5 membrane (concentrator; Amicon Corp., Danvers, MA). The protein content was determined by protein assay (Bio-Rad Laboratories).

To assess the purity of the affinity-purified *Drosophila* snRNPs, an aliquot of the *Drosophila* antigens (containing 150–250 μ g protein) was dialyzed to 0.1 times the normal Tris buffer. The sample was concentrated in a concentrator (Amicon Corp.) using a YM5 membrane until a final concentration of approximately 1 μ g/ml was achieved. The protein was mixed with four times the sample buffer and run on a 12.5 or 15% SDS polyacrylamide gel (Laemmli, 1970). Visualization of the protein was accomplished by either silver stain or Coomassie blue stain.

Ouchterlony Experiments and Counterimmunoelectrophoresis (CIE)

Drosophila crude extracts and purified snRNPs were used in a double

diffusion assay (Ouchterlony, 1958) with characterized SLE sera. CIE was performed as described by Kurata and Tan (1976).

ELISA and Inhibition ELISA

ELISA and inhibition ELISA were performed as described by Reichlin (1987). *Drosophila* heat-shocked and non-heat-shocked snRNPs or bovine snRNPs isolated from the affinity columns were used to coat 96 well polystyrene microtiter plates that were incubated for 2 h at room temperature or overnight at 4°C. The plates were washed three times with PBS with 0.05% Tween-20 (PBST) and then blocked with PBS containing 0.1% BSA for 1 h at room temperature. After washing three times with PBST, characterized SLE sera diluted in PBST with 0.1% BSA were used as the primary antibody and added to the wells and incubated for 2 h at room temperature. The plates were washed four times with PBST, after which goat anti-human IgG alkaline phosphatase-conjugated antibody at a 1:1,000 dilution in PBST with 0.1% BSA was added to all wells and incubated 2 h at room temperature. The plates were then washed four times with PBST and the substrate *p*-nitrophenyl phosphate added. At 30 min and 1 h after addition of the substrate, the microtiter plate was read at an absorbance of 405 nm on a scanner (Dynatech Laboratories, Inc., Alexandria, VA).

As a modification of the above procedure, the SLE sera were incubated for 2 h at room temperature or overnight at 4°C with varying concentrations of bovine nRNP or bovine Sm (100, 10, or 1 µg/ml) or PBS. The preincubated sera were then used as the primary antibody in the ELISA.

F(ab')₂ Purification and Isolation

Anti-Sm and Anti-nRNP specifically purified F(ab')₂ fragments were prepared as described by Gaither and Harley (1985) with the modifications discussed below.

To prepare anti-Sm F(ab')₂, bovine spleen extract (BSE) was depleted of nRNP by successive passages over an anti-nRNP affinity column. The nRNP content of the BSE was measured by its ability to inhibit anti-nRNP serum in an inhibition ELISA assay. When the inhibition was <10%, the BSE was considered nRNP depleted. This BSE was then applied to an anti-Sm affinity column and the column rinsed thoroughly with ACB. IgG from anti Sm serum was dialyzed to ACB and applied to the BSE-charged anti-Sm column. The column was then eluted with 3 M MgCl₂, and the fractions were monitored by absorbance at 280 nm. Pooled fractions were dialyzed to 0.2 M sodium acetate buffer, pH 4.2, and pepsin was digested for 18 h at 37°C. The digest was dialyzed to normal Tris buffer using 3,500-mol-wt cutoff dialysis tubing for 24 h at 4°C. The dialyzed digest was concentrated in a concentrator (Amicon Corp.) to final volume of 1–2 ml and applied to a Sephadex G-100 gel filtration column. The collected fractions were monitored by their absorbance at 280 nm and then tested in an ELISA for the presence of F(ab')₂ and/or Fc fragments.

Sandwich ELISA

Purified F(ab')₂ fragments with either anti-nRNP or anti-Sm specificity were used to coat 96-well polystyrene microtiter plates. The plates were washed with PBST and blocked for 1 h with 0.1% gelatin in PBST at room temperature. They were washed four times with PBST, and the antigen source was added and incubated for 2 h at room temperature or overnight at 4°C. The plates were again washed four times with PBST. The primary antibody, a characterized SLE serum, was diluted in PBS, added to the wells, and incubated for 2 h at room temperature. The plates were then washed four times with PBST. The secondary antibody, goat anti-human Fc conjugated with alkaline phosphatase, was diluted 1:8,000 in PBS and added to the plates. After a 2-h incubation at room temperature, the plates were washed four times with PBST, and substrate was added. The absorbance at 405 nm was monitored 30 min and 1 h after the addition of substrate.

To study specificity by inhibition analysis, the primary antibody was preincubated with bovine nRNP or bovine Sm at 100, 10, 1, and 0.1 µg/ml or PBS for 2 h at room temperature or overnight at 4°C. Such antibody with added antigen was used in place of the primary antibody in the assay described above for the inhibition analyses.

Results

Immunoprecipitation

To demonstrate that our human patient sera recognize con-

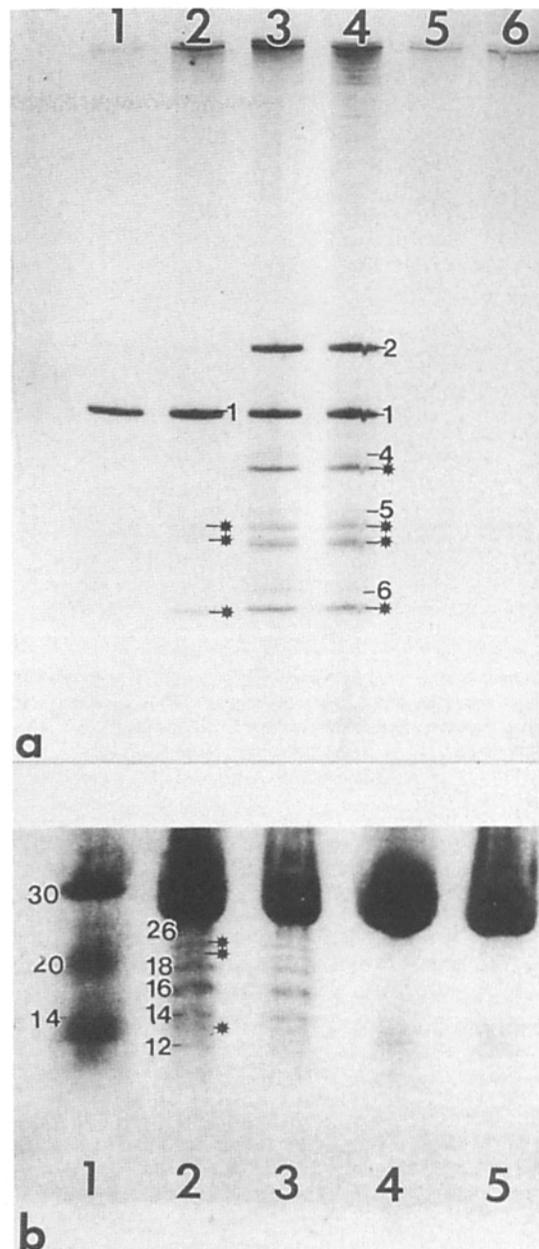


Figure 1. Immune precipitates from heat shocked and non-heat-shocked *Drosophila* embryos. (a) Small RNAs included in antibody precipitates from extracts of non-heat-shocked (lanes 1, 3, and 5) and heat-shocked (lanes 2, 4, and 6) embryos were fractionated on a 10% polyacrylamide gel. The antibody used was anti-nRNP (lanes 1 and 2); (lanes 3 and 4) anti-Sm; and (lanes 5 and 6) normal human serum. Small numbers indicate the respective U-RNAs; asterisks mark contaminating proteins detected by the silver stain (McNeilage and Whittingham, 1984). (b) Immunoprecipitated proteins from extracts of non-heat-shocked and heat-shocked embryos. Low molecular mass markers (lane 1); non-heat-shocked crude extract incubated with anti-Sm/nRNP-coupled beads (lane 2); and normal human serum-coupled beads (lane 4); heat-shocked crude extract incubated with anti-Sm/nRNP-coupled beads (lane 3) and normal human serum-coupled beads (lane 5). Sample buffer (two times the strength) was added to the washed beads and the mixture boiled for 10 min. The supernatant was loaded onto the gel and electrophoresed on an 8–25% SDS-polyacrylamide gradient gel. Proteins were visualized by Coomassie blue stain. The molecular masses of the proteins are indicated (kD). Asterisks indicate probable degradation products.

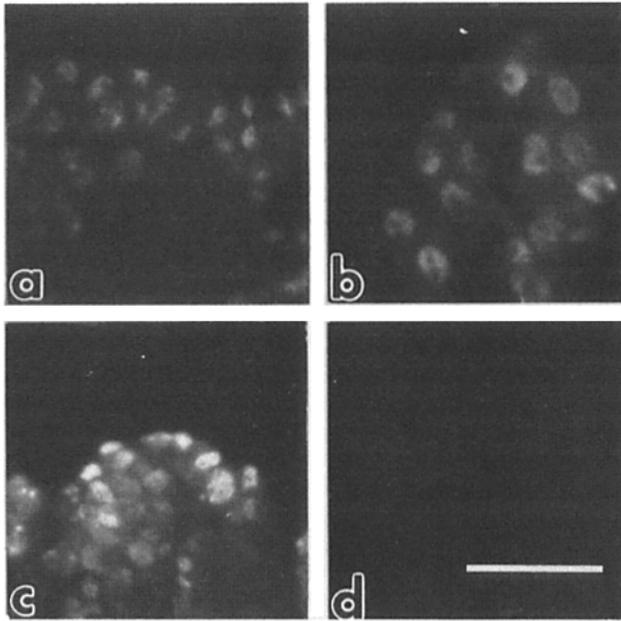


Figure 2. Fluorescent photomicrographs of sections of *Drosophila* embryos. Each section shown is stained by indirect immunofluorescence with a different serum. (a) Anti-Sm at a 1:100 dilution; (b) anti-nRNP at a 1:50 dilution; (c) anti-DNA at a 1:100 dilution; (d) normal human serum at a 1:100 dilution. All fluorescent micrographs were taken and printed under identical conditions, except that photomicrograph (a) was printed with a 10% reduction in exposure time. Bar, 0.02 mm.

served antigens found in *Drosophila melanogaster*, as previously demonstrated by Mount and Steitz (1981), *Drosophila* snRNP particles were immunoprecipitated. Two characterized SLE sera (see Materials and Methods) were used, as well as a normal human serum control. *Drosophila* embryo extracts were prepared and incubated with sera. Following this incubation, complexes were precipitated and RNAs extracted and separated by gel electrophoresis. The results of this experiment are shown in Fig. 1 a, which demonstrates that anti-nRNP sera are specific for the U1-snRNP of *Drosophila*, while anti-Sm sera recognize U1, U2, U4, U5, and U6 snRNPs of *Drosophila*. Normal human serum does not recognize the snRNPs.

The proteins of the *Drosophila* particles have been immunoprecipitated by SLE sera and subjected to SDS-PAGE (Fig. 1 b). Five proteins are immunoprecipitated, with molecular masses of 26, 18, 16, 14, and 12 kD. From immunoblotting with crude extract, we have shown that the two larger proteins are recognized by nRNP antigen and the three smaller proteins are reactive with Sm sera (data not shown).

Indirect Immunofluorescence

Drosophila embryo sections were stained by indirect immunofluorescence to demonstrate the nuclear staining pattern of the SLE sera (Fig. 2). The sections were stained with anti-Sm serum, anti-nRNP serum, anti-DNA serum, and normal human serum as indicated in the legend. The normal human serum was negative; the three other sera stained nuclei.

Indirect immunofluorescence was carried out with frozen

sections of 0–24-h *Drosophila* embryos (Fig. 3). The embryos were either embedded without heat shock immediately after collection (Fig. 3, a and e), heat shocked for 15 min at 33°C (Fig. 3, b and f), heat shocked for 15 min at 33°C and allowed to recover 2 h before a 15-min treatment at 37°C (Fig. 3, c and g), or heat shocked for 15 min at 37°C (Fig. 3, d and h) before embedding. Experimental sections shown in Fig. 3 (a–d) were incubated with an anti-Sm serum (1:100) for 20 min at room temperature. The sections were subjected to indirect immunofluorescence. Fig. 3 a illustrates a section of a 0–24-h non-heat-shocked *Drosophila* embryo, showing positive fluorescence. Fig. 3 b shows an embryo subjected to a mild 33°C, 15-min treatment, while Fig. 3 c depicts an embryo given this mild heat shock treatment 2 h before the more severe (37°C, 15 min) exposure. Both of these treatments resulted in no loss of fluorescence and were modeled after experimental protocols designed by Yost and Lindquist (1986) to test the effects of heat shock on splicing activity. Note that the mild treatment in fact appears to protect the embryo from the loss of antigen recognition during subsequent severe conditions. Fig. 3 d shows a 0–24-h heat-shocked *Drosophila* embryo treated at 37°C for 15 min. The loss of fluorescence is easily seen in this severe heat shock treatment (Fig. 3 d). 100% of the embryos examined showed a loss of fluorescence comparable to that illustrated in Fig. 3 d. An anti-nRNP serum (1:100) gave positive and equal results on both heat-shocked and non-heat-shocked sections, regardless of the treatment regimen (Fig. 3, e–h). These data suggest the loss of antigen recognition in the heat-shocked sections for the antibodies present in the anti-Sm sera. No detectable difference between heat-shocked and non-heat-shocked sections was observed with the anti-nRNP sera. The staining pattern reflects the higher concentration of nuclei around the periphery of the embryos.

The panel of photographs shown in Fig. 4 illustrates a series of control experiments for the immunofluorescent data presented above. Sections from non-heat-shocked embryos were incubated with anti-Sm serum (Fig. 4 a), anti-nRNP serum (Fig. 4 b), anti-DNA serum (Fig. 4 c), and normal human serum (Fig. 4 d). Sections incubated with anti-Sm serum, anti-nRNP serum, and anti-DNA serum were positive, while the normal human serum sections were negative. The positive control (anti-DNA serum) and the negative control (normal human serum) were unaffected by heat shock (data not shown).

To demonstrate the specificity of the SLE sera being used, the sera were preincubated with 200 µg/ml affinity-purified bovine Sm or nRNP antigen (Fig. 4, e–h). The final dilution of the antibody was maintained at 1:100. Fig. 4 e depicts a non-heat-shocked 0–24-h *Drosophila* embryo section that was treated with the preincubated, inhibited anti-Sm serum. Fig. 4 (f, g, and h) depict non-heat-shocked 0–24-h *Drosophila* embryo sections incubated with anti-nRNP, anti-DNA, and normal human sera, each of which had been preabsorbed with affinity-purified bovine nRNP antigen. As shown by the photographs, the bovine Sm was able to inhibit the anti-Sm serum and thus drastically reduce the fluorescence levels (Fig. 4 e). The preincubation of anti-nRNP serum with nRNP antigen caused greatly reduced fluorescence in such sections (Fig. 4 f). Both the positive (anti-DNA) and negative (normal human serum) controls were treated identically to the inhibited anti-nRNP sections and were unchanged by

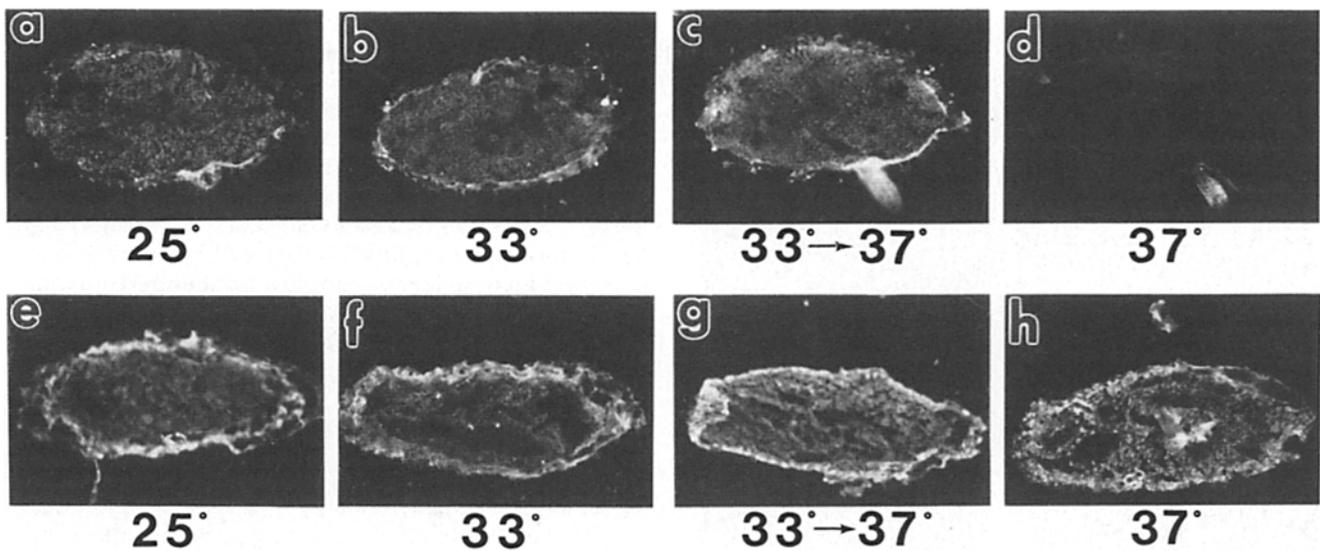


Figure 3. Immunofluorescence patterns of non-heat-shocked and heat-shocked *Drosophila* 0-24-h embryo sections. *a*, *b*, *c*, and *d* were incubated with anti-Sm serum (1:100 dilution); *e*, *f*, *g*, and *h* were incubated with anti-nRNP serum (1:100 dilution). Both preparations were then stained with FITC-conjugated anti-human IgG antibodies. 25° (*a* and *e*) indicates the embryos were raised at 25°C and had no heat shock treatments. 33° (*b* and *f*) indicates the embryo was heat shocked for 15 min at 33°C before embedding. 33°→37° (*c* and *g*) indicates that the embryos were heat shocked for 15 min at 33°C, allowed to recover for 2 h at room temperature, and then subjected to a 15 min heat shock at 37°C before embedding. 37° (*d* and *h*) indicates that the embryos were heat shocked for 15 min at 37°C before embedding. Bar, 0.1 mm.

the preincubation of serum with bovine nRNP (Fig. 4, *g* and *h*). These experiments strongly support the idea that the fluorescence of whole anti-Sm and anti-nRNP sera are due to their content of antibody to the Sm and nRNP antigens respectively.

To test the specificity of the above procedures further, the experiments were repeated with specifically purified F(ab)₂ fragments with anti-Sm or anti-nRNP activity. These experiments yielded results identical to those presented above for

whole sera: the uninhibited anti-Sm F(ab)₂ resulted in bright apple green sections from non-heat-shocked embryos, heat shocked (33°C, 15 min) embryos, and pretreated embryos (33°C, 15 min; recovery 2 h; 37°C, 15 min). Heat shocked embryos (37°C, 15 min) showed a loss of fluorescence (data not shown). F(ab)₂ fragments were inhibited by their respective affinity-purified bovine antigens, resulting in a loss of fluorescence in all previously positive sections. This same procedure was performed with F(ab)₂ of anti-nRNP speci-

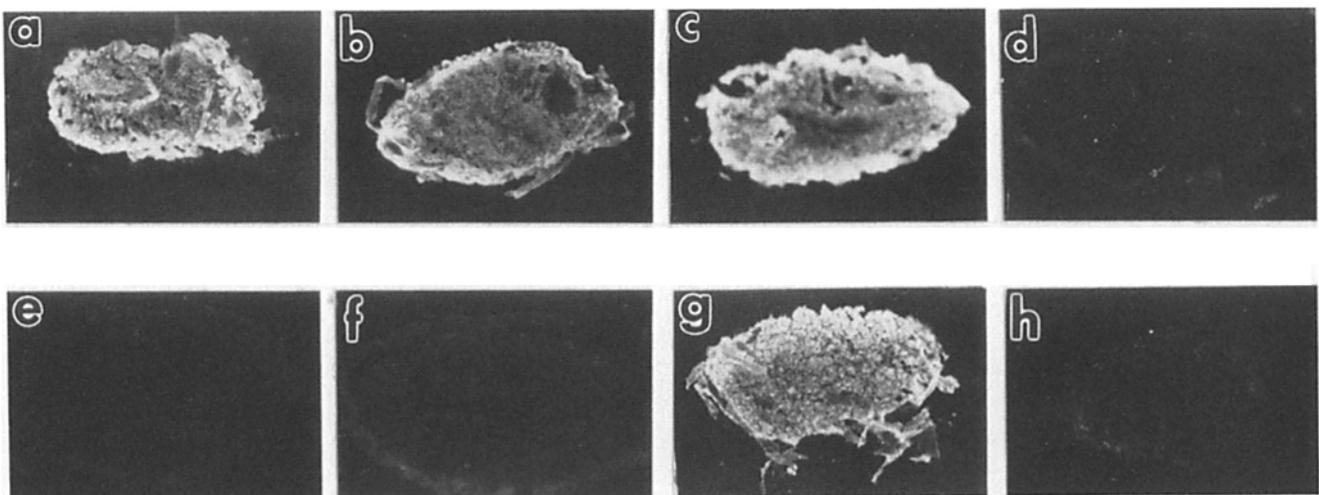


Figure 4. Immunofluorescence patterns of non-heat-shocked *Drosophila* 0-24-h embryo sections. *a*, *b*, *c*, and *d* were incubated with the following sera: (*a*) anti-Sm serum; (*b*) anti-nRNP serum; (*c*) anti-DNA serum; and (*d*) normal human serum. *e*, *f*, *g*, and *h* were incubated with the corresponding sera, each of which had been preincubated with (*e*) 200 µg/ml of bovine Sm, or (*f*, *g*, and *h*) 200 µg/ml of bovine nRNP. All sera were diluted 1:100.

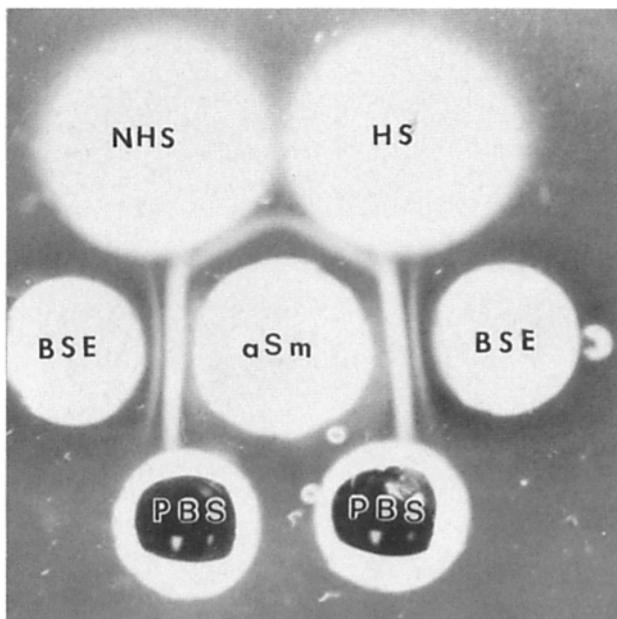


Figure 5. Ouchterlony double immunodiffusion comparison of heat-shocked and non-heat-shocked *Drosophila* 0-24-h embryo extracts. *NHS*, non-heat-shocked *Drosophila* 0-24-h embryo extract (32 mg/ml protein); *HS*, heat-shocked *Drosophila* 0-24-h embryo extract (32 mg/ml protein); *BSE*, bovine spleen extract; *αSm*, anti-Sm serum 1:4.

ficity. Both heat-shocked and non-heat-shocked sections gave positive fluorescence before inhibition. The anti-nRNP specifically purified $F(ab)_2$ could be inhibited by bovine nRNP to give a dull, dark fluorescence on both heat-shocked and non-heat-shocked sections (data not shown). The specific inhibition of the nRNP and Sm fluorescent signals by affinity purified antigens and the identical patterns of fluorescence with purified $F(ab)_2$ fragments of both specificities argue strongly that the fluorescent signal is specific for snRNP particles.

To investigate the antigenic differences between heat-shocked and non-heat-shocked embryonic sections that were observed in the indirect immunofluorescence experiments, we carried out a series of experiments designed to characterize the antigens in embryo extracts. These experiments are described below.

Ouchterlony and CIE

To test whether the difference noted in the indirect immunofluorescence was also observed in diffusion assays and to assess this difference with a known standard, Ouchterlony assays and CIE were performed. *Drosophila* crude extracts prepared from heat-shocked and non-heat-shocked 0-24-h embryos were tested in a double diffusion assay (Ouchterlony, 1958), as shown in Fig. 5. Bovine spleen extract (BSE) was used as a positive control and PBS as a negative control. When SLE serum with anti-Sm specificity was tested, the heat-shocked and non-heat-shocked embryo extracts gave lines of identity with each other and shared a reaction of partial identity with BSE. When anti-nRNP specific serum was used, no precipitin lines were formed with either heat-shocked or non-heat-shocked crude extracts.

The above assays were repeated with affinity-purified *Drosophila* snRNPs, with identical results for anti-Sm (data not shown). Once again, the *Drosophila* nRNP antigens failed to form a precipitin line in the anti-nRNP assays (data not shown).

CIE, which is slightly more sensitive than the Ouchterlony double diffusion assay, gave identical results. These results suggest that the *Drosophila* Sm antigens were reactive with human anti-Sm sera but were antigenically deficient with respect to the bovine snRNP particles. To examine these relationships quantitatively, ELISA assays were performed to assess the differences between the heat-shocked and non-heat-shocked *Drosophila* snRNPs.

Characterization of *Drosophila* Sm Antigens by ELISA Assays

We wished to compare the ability of each of the three antigens (non-heat-shocked *Drosophila* Sm, heat-shocked *Drosophila* Sm, and bovine Sm) to react with the human autoimmune sera by inhibition ELISA. Before carrying out these experiments, the level of affinity-purified Sm antigen that saturates the binding sites on the microtiter dish must be determined. With the use of varying Sm antigen concentrations versus a constant dilution of SLE serum of anti-Sm specificity, we determined that even at concentrations as low as 10 $\mu\text{g/ml}$, the binding sites of the microtiter plate appear to be saturated. To ensure complete saturation of all binding sites, we chose to coat with 15 $\mu\text{g/ml}$. The inhibition assays also require the determination of a useful dilution of anti-Sm serum versus a constant antigen concentration. There were no detectable differences between heat-shocked, non-heat-shocked, or bovine Sm when titered against anti-Sm specific serum. These data were used to select a serum dilution for the inhibition study presented below.

For inhibition analysis, plates were coated with affinity-purified heat-shocked Sm, nonheat-shocked Sm, or bovine Sm. A fixed dilution of anti-Sm was blocked with increasing amounts of affinity-purified *Drosophila* heat-shocked Sm (Fig. 6 A), *Drosophila* non-heat-shocked Sm (Fig. 6 B) and bovine Sm (Fig. 6 C), respectively. Fig. 6 (A and B) are equivalent to each other and show essentially identical inhibition curves. This demonstrates that the quantity of Sm antigen is not reduced by heat shock treatment. When bovine Sm is the antigen bound to the plate, neither *Drosophila* heat-shocked nor *Drosophila* non-heat-shocked Sm are able to inhibit the entire reaction and indeed, only 25-33% of the reactivity is inhibitable. This is a quantitative demonstration of the partial identity reaction seen in the qualitative Ouchterlony analysis (Fig. 5). Thus, there is a subpopulation of anti-Sm antibodies that are able to react with bovine Sm, but not with *Drosophila* Sm. Fig. 6 C shows that whatever the coating antigen (*Drosophila* heat-shocked Sm, *Drosophila* non-heat-shocked Sm, or bovine Sm), the bovine Sm, as expected, is able to inhibit all three efficiently and in equivalent fashion. Therefore, the anti-Sm antibody subpopulation that can recognize the *Drosophila* Sm antigen also binds the bovine antigen with equal affinity.

Characterization of *Drosophila* nRNP Antigens by ELISA Assays

Experiments similar to the anti-Sm inhibition ELISA assays

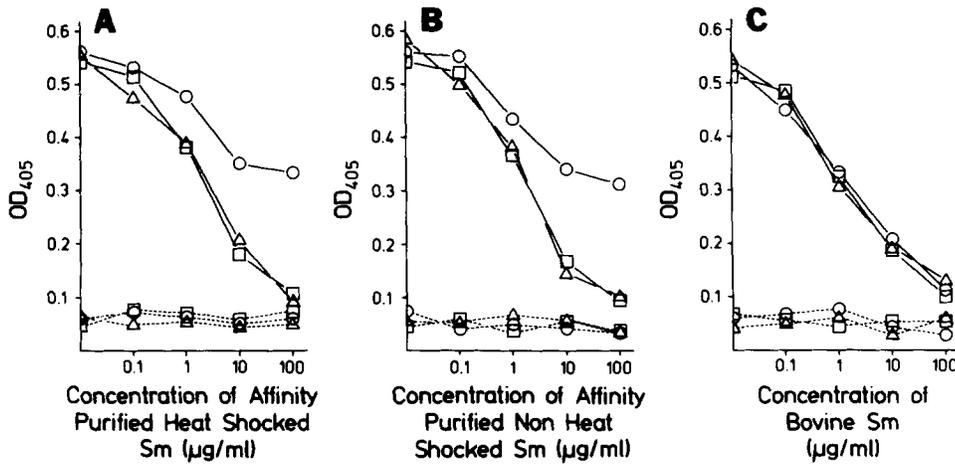


Figure 6. Inhibition ELISA using affinity-purified 0–24-h *Drosophila* heat-shocked and non-heat-shocked Sm antigen and bovine Sm antigen. The microtiter plates were coated with 15 mg/ml of heat-shocked *Drosophila* Sm (Δ); non-heat-shocked *Drosophila* Sm (\square); or bovine Sm (\circ). Anti-Sm serum at a final dilution of 10^{-4} (—) and normal human serum at a final dilution of 10^{-3} (---) were incubated with varying concentrations of heat-shocked *Drosophila* Sm (A); non-heat-shocked *Drosophila* Sm (B); or bovine Sm (C), and then incubated in the coated wells. After washing, goat anti-human alkaline phosphatase-conjugated antibody was added to all wells. The reaction of alkaline phosphatase with substrate results in the development of a yellow color that is read at 405 nm.

were performed with anti-nRNP to assess and quantify the differences, if any, among heat-shocked and non-heat-shocked *Drosophila* nRNP and bovine nRNP. Before carrying out these assays, we determined that an antigen concentration of $\sim 5 \mu\text{g/ml}$ provided saturation of all binding sites of the microtiter plate wells; we chose to coat with $15 \mu\text{g/ml}$ to ensure saturation. We also carried out a titration of anti-nRNP serum dilutions versus a constant antigen concentration. While we detected no differences between the heat-shocked and non-heat-shocked *Drosophila* nRNP antigens, there was, however, a large difference between the bovine and *Drosophila* antigens. Even at concentrations that saturate binding, the

SLE sera of anti-nRNP specificity did not recognize the *Drosophila* antigen as effectively as the bovine antigen. This difference can be explained by one of two hypotheses: (a) a subpopulation of antibodies exists within the serum that recognize the bovine nRNP antigen and not the *Drosophila* antigen; or (b) an affinity difference exists such that the SLE autoantibodies recognize the *Drosophila* antigens with a lower affinity than the bovine antigens. The inhibition assays presented below will distinguish between these two possibilities.

Inhibition assays were carried out by coating the plates with affinity-purified *Drosophila* heat-shocked nRNP, non-

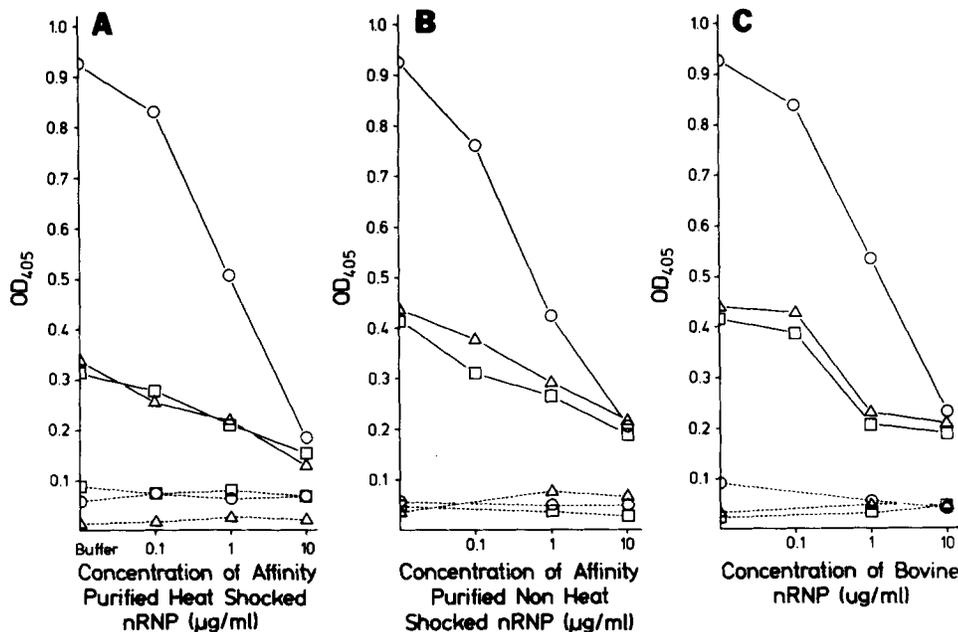


Figure 7. Inhibition ELISA using affinity-purified 0–24-h *Drosophila* heat-shocked and non-heat-shocked nRNP and bovine nRNP. The microtiter plates were coated with 15 mg/ml of heat-shocked *Drosophila* nRNP (Δ); non-heat-shocked *Drosophila* nRNP (\square); and bovine nRNP (\circ). Anti-nRNP serum at a final dilution of 10^{-3} (—) and normal human serum at a final dilution of 10^{-3} (---) were incubated with varying concentrations of heat-shocked *Drosophila* nRNP (A), non-heat-shocked *Drosophila* nRNP (B), or bovine nRNP (C), and then incubated in the coated wells. After washing, goat anti-human alkaline phosphatase-conjugated antibody was added to all wells. The reaction of alkaline phosphatase with substrate results in the development of a yellow color that is read at 405 nm.

heat-shocked nRNP, or bovine nRNP. Then a fixed anti-nRNP serum dilution was incubated with increasing amounts of affinity-purified *Drosophila* heat-shocked nRNP (Fig. 7 A), *Drosophila* non-heat-shocked nRNP (Fig. 7 B) and bovine nRNP (Fig. 7 C), respectively. Surprisingly, *Drosophila* nRNP from either heat-shocked or non-heat-shocked 0–24-h embryos and the bovine nRNP all inhibit to the same levels. Therefore, the differences in reactivity between *Drosophila* and bovine antigens suggest a stronger affinity of the nRNP antibodies for the bovine antigen. The Ouchterlony data presented above indicated that the *Drosophila* nRNP antigen was unable to form a precipitin line with the anti-nRNP serum. The inability to form a precipitin line is therefore because of the apparent affinity difference between the antibodies for *Drosophila* and bovine nRNP antigens.

Sandwich ELISAs and their inhibition counterpart were performed to assess and to quantify any differences between heat-shocked and non-heat-shocked *Drosophila* snRNPs. The results of both the titration and inhibition of antibody binding to Sm and nRNP as measured in sandwich assays were identical to the immunochemical data presented in the previous section.

Restoration of Indirect Immunofluorescence

In an attempt to reconcile the differences between the indirect immunofluorescence data and the immunochemical data, we decided to preincubate heat-shocked sections with phosphate buffer containing varying salt concentrations (Fig. 8). ddH₂O or 0.02 M phosphate with 0.15, 0.5, or 1.0 M NaCl were used. The sections were then rinsed and the primary antibody applied. The primary antibodies included anti-Sm serum (1:100) (Fig. 8, a–d); anti-nRNP serum (1:100) (Fig. 8, e–h); anti-DNA serum (1:10 and 1:100) (Fig. 8, i–l); and normal human serum (1:10 to 1:100) (Fig. 8, m–p).

Fig. 8, a–d show that the heat-shocked 0–24-h embryo sections preincubated with no salt and with 0.15 M salt before incubation with anti-Sm sera remained negative. However, the heat-shocked sections preincubated with phosphate buffer with 0.5 M and 1 M NaCl regained positive fluorescence, with the following limitations: (a) only 60–70% of the total embryos on the slide gave positive results; (b) in each individual embryo viewed, the entire embryo was either a bright, apple green (fluorescence restored) or a dull, dark green (fluorescence not restored).

To investigate whether the restoration was occurring at a particular embryonic age, the embryos were examined under visible light microscopy and staged for developmental age. The embryos that failed to have their fluorescence restored were not of a particular age, and, in fact, represented all stages of 0–24-h embryonic development. Identical results were obtained for both a 15-min and a 1-h heat shock treatment at 37°C.

Several control experiments were essential in this experiment. First, since snRNPs are soluble, a loss of fluorescence in the sections treated with anti-nRNP would indicate a loss of antigen. However, the sections treated with anti-nRNP serum were all unchanged with fluorescence equivalent among the various treatments (Fig. 8, e–h). Thus, a loss of antigen did not appear to occur. Second, if the salt pretreatment affected the antibody–antigen interaction, it would be evident by either loss of fluorescence on the anti-DNA serum-treated sections or enhancement of fluorescence on the normal human serum-treated sections. The anti-DNA treated sections

showed identical fluorescence for all preincubation fluids with the exception of the phosphate buffer with 1 M NaCl. In this case, there was a slight decrease in fluorescence in two out of three trials. The sections treated with normal human serum were all negative.

Discussion

The studies presented here describe the immunological characterization of *Drosophila* snRNPs as recognized by the sera of SLE patients with anti-snRNP activity. In an indirect immunofluorescence study of *Drosophila* tissue sections, a dramatic loss of fluorescence for anti-Sm sera was observed if the embryos were heat shocked for 15 min at 37°C before embedding and sectioning. However, a mild heat shock treatment (33°C for 15 min) did not result in this loss of fluorescence. In fact, the mild heat shock administered before the 37°C heat shock protected against the subsequent loss of fluorescence in the sections. We carried out further immunological characterization of snRNPs in extracts from heat-shocked and non-heat-shocked embryos and found no antigenic differences between the snRNPs from heat shocked versus non-heat-shocked embryo extracts. It is evident that a striking dichotomy exists. The indirect immunofluorescence data demonstrate a difference between heat-shocked and non-heat-shocked Sm antigens, whereas the immunochemical data indicate identity. A major difference between the two experimental protocols is the processing required to produce crude extracts for use in immunological tests. The extracts are subject to 0.5 M salt before reaction with antibody, while the frozen embryo sections are only exposed to 0.15 M salt before antibody binding (see Materials and Methods). Consequently, we tested the hypothesis that a heat shock-induced antigenic alteration might revert in higher salt concentrations. We found that preincubation of the embryo sections with a salt concentration equal to that used for the extracts restored fluorescence, explaining the immunological identity of heat-shocked and non-heat-shocked snRNPs. The ability of 0.5 and 1.0 M NaCl concentrations to restore fluorescence in heat-shocked sections suggests a noncovalent alteration of the antigen.

We used the experimental design of Yost and Lindquist (1986) to determine whether the loss of indirect immunofluorescence of Sm antigens that we observed parallels their observation of loss of splicing activity. Indeed, both splicing activity and antigenic recognition are lost upon the administration of the severe heat shock; in addition, the mild heat shock pretreatment provides protection from both loss of splicing activity and loss of Sm antigenic recognition during the subsequent severe heat shock. These experimental results provide evidence supporting a link between the heat shock-induced alteration of *Drosophila* Sm antigens presented in this paper and the loss of splicing activity observed by Yost and Lindquist (1986).

While there are several possible interpretations of these data, the simplest hypothesis is that splicing components undergo a conformational change upon exposure to heat shock, resulting in an antigenically inactive and nonfunctional state. Thus, the salt incubations release the components from this altered conformation and again permit recognition by SLE antibodies. We further suggest that the induction of heat shock proteins (hsps) by the mild heat pretreatment stabilizes the splicing components in their native configuration so that

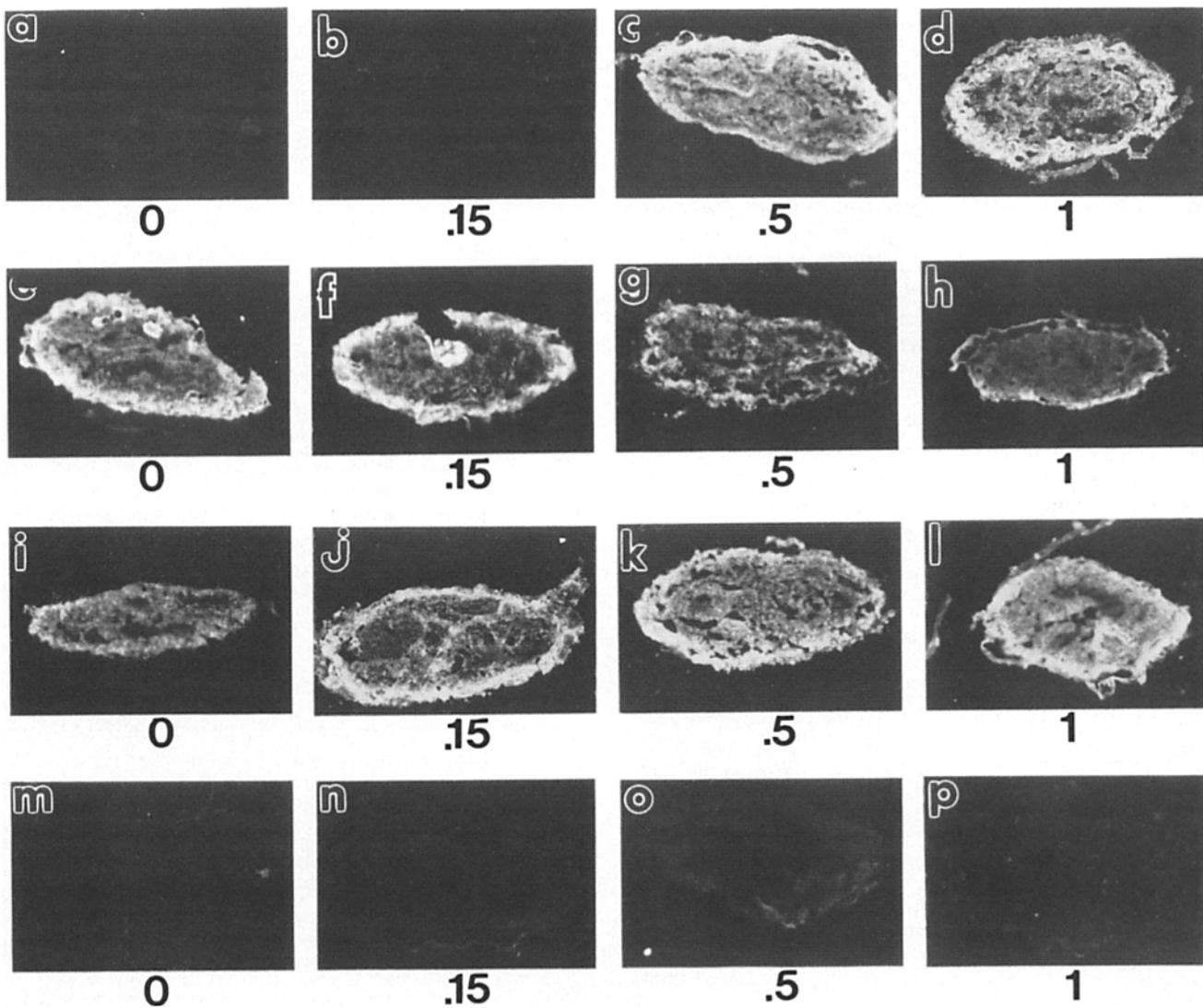


Figure 8. Restoration of fluorescence by brief salt washes. The sections have all been subjected to a 37°C, 15-min heat shock before embedding and sectioning. *a, e, i, and m* (0) were incubated with phosphate buffer containing no NaCl. *b, f, j, and n* (.15) were incubated with phosphate buffer containing 0.15 M NaCl. *c, g, k, and o* (.5) were incubated with phosphate buffer containing 0.5 M NaCl. *d, h, l, and p* (1) were incubated with phosphate buffer containing 1.0 M NaCl. *a, b, c, and d* were incubated with anti-Sm serum (1:100) after the salt washes. *e, f, g, and h* were incubated with anti-nRNP serum (1:100) after the salt washes. *i, j, k, and l* were incubated with anti-DNA serum (1:100) after the salt washes. *m, n, o, and p* were incubated with normal human serum (1:100) after the salt washes. All the sections were then stained with FITC-conjugated anti-human IgG antibodies.

they do not lose fluorescence in a subsequent severe heat shock.

Results of the experiments of Yost and Lindquist (1986) provide support for this hypothesis. In their carefully designed study of the effects of heat shock on splicing, they observed that cycloheximide does not affect the heat shock-induced block of RNA splicing. This result suggests that the synthesis of hsp's is not necessary for the block in splicing. In addition, they found that synthesis of hsp's is required for the protective effect of the mild heat shock pretreatment. Assuming that the antigenic alteration we detect is a reflection of changes that result in the splicing block, a direct role for the hsp's in the antigenic alteration may be unlikely.

Of the hsp's, hsp70 moves to the nucleus upon heat shock (Velazquez and Lindquist, 1984). Subsequent research has suggested that members of the hsp 70 gene family act as "chaperone" proteins (Pelham, 1988), which maintain the

native configuration of proteins as they move from one cellular compartment to another (Deshaies et al., 1988; Chirico et al., 1988). The small hsp's associate with the vimentin-based cytoskeleton that collapses around the nucleus upon heat shock administration (Leicht et al., 1986), though hsp 23 has also been reported to be localized in the nucleolus (Duband et al., 1986). Hsp 82 exhibits the properties of a soluble cytoplasmic protein (Tanguay, 1985). Therefore, of the hsp's, hsp 70 is the most likely candidate for conferring the protective effect of the mild heat shock pretreatment. Of course, indirect effects of heat shock offer additional possible mechanisms both for the initial antigenic alteration and the protective effect of the mild heat shock pretreatment.

Yost and Lindquist (1986) proposed that the block in splicing might have evolved as a mechanism to give heat shock transcripts an immediate advantage under physiologically stressful conditions. Of the genes that respond to heat shock,

only two produce a transcript that is spliced. The heat shock responsive gene at locus 93 D does not appear to encode a protein (Garbe et al., 1986), but produces a spliced transcript. The sole heat shock gene encoding a protein (hsp83) that possesses an intron is the gene chosen by Yost and Lindquist (1986) to demonstrate the heat shock-induced loss of splicing activity. Though hsp 83 mRNAs are rapidly transcribed, there is a lag in the appearance of the protein during a severe heat shock because of the processing delay. Genes encoding heat shock cognate proteins, which are closely related to hsps, are expressed at normal temperatures and do contain introns (Ingolia and Craig, 1982).

Autoantibodies in human lupus sera are able to recognize splicing protein-RNA complexes from the phylogenetically distant organism, the fruit fly. This antigenic reactivity has permitted us to characterize the proteins that are part of the *Drosophila* snRNP particles. We have identified five proteins that can be precipitated with anti-snRNP sera. Two of these are reactive with anti-nRNP antibodies, and the three smaller proteins are recognized by anti-Sm sera. In previous studies, Weiben and Pederson (1982) identified two [³⁵S]methionine proteins of 26 and 14 kD that were precipitated by sera of anti-nRNP specificity. A reduced amount of methionine in the proteins that were not detected might explain this result. The 14-kD protein they detected may be the same protein that we have identified as an Sm antigen. Because Sm antigens are present on nRNP particles, the protein will appear in immunoprecipitates. Wooley et al. (1982) fractionated *Drosophila* snRNPs on isokinetic sucrose gradients, and were able to detect proteins of 26 and 18 kD by challenge with SLE antiserum. Because these proteins are those that we have identified as reactive with anti-nRNP sera, it is possible that the serum they used had a high content of anti-nRNP.

Conservation of the antigens between the phylogenetically distinct groups is strongly supported by these data. However, the antigens are not identical. The proteins recognized by anti-nRNP have a different amino acid sequence or a slightly different three-dimensional conformation such that the antigen-antibody affinity is greatly lowered. The epitopes recognized by the antibodies of anti-Sm sera appear to have equal affinity for *Drosophila* and bovine sources for a major portion of the anti-Sm antibodies. However, the *Drosophila* Sm and extracts are unable to inhibit completely the binding of anti-Sm sera to bovine Sm. This strongly implies that the sera used have an antibody subpopulation that does not recognize the *Drosophila* antigen but does recognize the bovine antigen. In addition, these experiments show that the quantity of Sm antigen in heat-shocked embryos is equal to the quantity in nonheat-shocked embryos. Therefore, loss of immunofluorescent signal is not because of breakdown of the antigen itself. Thus, the human autoantibodies of Sm specificity recognize at least two mammalian epitopes: one conserved epitope that is affected by heat shock, and another epitope that is present in mammals but can no longer be detected on the *Drosophila* antigen.

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