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Sodium Salicylate Decreases Intracellular ATP, Induces Both Heat Shock Factor Binding and Chromosomal Puffing, but Does Not Induce hsp 70 Gene Transcription in *Drosophila**

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Sodium salicylate has long been known to be an inducer of the heat shock puffs and presumably heat shock gene transcription in the polytene chromosomes of *Drosophila* salivary gland cells. Stress-induced transcription of the heat shock genes is mediated by the transcription factor known as Heat Shock Factor (HSF). In yeast, sodium salicylate has been reported to induce the DNA binding of HSF but not heat shock gene transcription itself, and similar findings have been reported in human cells. This apparent discrepancy in the induction of certain aspects of the heat shock response between these organisms prompted us to carefully reexamine the induction of the heat shock response in *Drosophila* salivary gland cells of third instar larvae and *Drosophila* tissue culture (SL2) cells. Sodium salicylate (3–30 mM) decreases intracellular ATP levels in SL2 cells and induces HSF binding activity in SL2 and salivary gland cells in a dose-dependent manner. Despite the induction of HSF binding and heat shock puffs in polytene chromosomes, we found no evidence for increased hsp 70 gene transcription suggesting that chromosomal puffing and gene transcription may be separable events. Salicylate did not induce the HSF hyperphosphorylation that is normally associated with HSF activation. Furthermore, salicylate (30 mM) prevented heat-induced hyperphosphorylation of HSF and hsp 70 gene transcription indicating that salicylate's inhibitory effect on hsp 70 transcription may be independent of its effect on HSF binding activity. We propose that the reduction in intracellular ATP caused by the addition of salicylate likely plays a role in the activation of HSF binding and the inhibition of both HSF hyperphosphorylation and hsp 70 gene transcription.

One of the most widely administered family of drugs is the anti-inflammatory salicylates. Although many aspects of their action have been characterized, such as their effect on prostaglandin biosynthesis (1), a total understanding of their pharmacological mechanism is not known.

Salicylate has been demonstrated also to have both positive and negative effects on the transcriptional activity of a number of genes. In plants, salicylate can accumulate in disease ridden organisms and is believed to be involved in the induction of pathogenesis related genes (2). In contrast, other genes expressed during leaf wounding apparently are inhibited by salicylate (3). In human cells, salicylate has been shown to inhibit nuclear factor κB activation and transcription of genes regulated by this transcription factor (4).

Over 30 years ago Ritossa reported that heat (30 °C), 1 mM 2,4-dinitrophenol, and 10 mM sodium salicylate induced the puffing of a specific set of loci in the polytene chromosomes of *Drosophila* salivary gland cells (5, 6). These "heat shock" puffs have always been assumed to be the site of active transcription of the heat shock genes because [^{3}H]uridine is incorporated at these sites (7, 8) and heat shock gene cDNAs hybridize to RNA found at the puff sites (9). Furthermore, the addition of RNA synthesis inhibitors blocks the induction of the heat shock puffs (for review, see Ref. 8). Since the discovery of the heat shock puffs, the heat shock response has been described in every organism examined and a multitude of different "stress" inducers have been identified (for review, see Ref. 10). In eukaryotic cells, the transcriptional response to stress is mediated through a transcription factor known as heat shock factor (HSF)[1] (for reviews, see Refs. 11–14). In metazoans, stress induces a transformation of HSF from a monomer (for HSF1) to a trimer; and it is this latter form that binds to a specific DNA sequence known as the HSE (for review, see Ref. 15). Binding of HSF to the HSE is presumed to be required for heat shock gene transcription, but it is not clear if it is sufficient.

In *Drosophila* tissue culture cells (Schneider Line 2, SL2), heat, 2,4-dinitrophenol, and salicylate treatments have been shown to induce the high affinity binding of HSF *in vivo* (16). Experiments using human cells have indicated that salicylate is capable of inducing both HSF binding and heat shock gene transcription at certain concentrations, while other concentrations induce HSF binding only (17, 18). The situation is somewhat different in yeast where sodium salicylate stimulates HSF binding, but transcription of heat shock genes does not occur. Surprisingly, addition of salicylate prevents the induction of heat shock genes by heat in this organism (19).

In this study, we show that sodium salicylate (3–30 mM) induces activation of HSF binding activity in both salivary gland cells and SL2 tissue culture cells. Puffing of the heat shock gene loci occurs in salivary glands exposed to 10 mM salicylate, and to a lesser extent, with exposure to 3 mM salicylate. No puffing occurs with 30 mM salicylate treatments, and this concentration prevents puffing of the heat shock gene loci

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[1] The abbreviations used are: HSF, heat shock factor; Bis-Tris, bis-(2-hydroxyethyl)iminotris(hydroxymethyl)methane; EMSA, electrophoretic mobility shift assay; HSE, heat shock element; hsp, heat shock protein; SL2, Schneider line 2; TB1, transcription buffer 1; TBE, Tris-borate-EDTA; PAGE, polyacrylamide gel electrophoresis.

by heat. However, salicylate treatments (3–30 mM) do not induce transcription of the hsp 70 heat shock genes in salivary glands nor in SL2 cells as assayed by primer extension analysis. These results suggest that chromosomal puffing and gene transcription are likely separable events. In addition, and similar to what is seen in yeast, salicylate (30 mM) prevents heat-induced transcription of the heat shock genes.

It is not known how salicylate activates HSF binding, but models have been proposed which indicate that the lowering of intracellular pH (19) or the accumulation of newly synthesized aberrant proteins (17, 18) might be involved in the activation process. Here we show that, in *Drosophila* cells, salicylate dramatically inhibits cellular ATP levels and prevents HSF hyperphosphorylation normally seen with heat-induced activation of HSF. We propose that sodium salicylate interferes with oxidative respiration resulting in decreased cellular ATP. A model as to how lowered ATP levels might activate HSF binding and prevent transcription of the heat shock genes is discussed.

EXPERIMENTAL PROCEDURES

Salivary Gland Treatments and Chromosome Squashes—Salivary glands were dissected from wandering third instar "Oregon R" *Drosophila melanogaster* larvae in a drop of modified TB1 buffer (15 mM HEPES, pH 6.8, 80 mM KCl, 16 mM NaCl, 5 mM $MgCl_2$, 1% polyethylene glycol 6000) (20, 21). Only glands exhibiting puff stage 1 morphology (as described in Ref. 21) were used in experiments. Experiments were performed in depression slides kept in humidified chambers. Glands were first incubated in TB1 for 60 min at room temperature. One lobe of each pair was then transferred to a depression slide containing the appropriate concentration of sodium salicylate dissolved in TB1; the other lobe remained in TB1 as a control. For experiments involving heat shock, glands were transferred to depression slides containing sodium salicylate in TB1, or TB1 alone, in a humidified container kept partially submerged in a water bath. A temperature probe inside the container allowed for monitoring of the temperature, which was maintained at 36.5 °C for heat shock. After the appropriate incubation time, experimental and control lobes were fixed immediately in 3.7% formaldehyde, 50% acetic acid, and squashed on microscope slides under coverslips treated with Sigmacote (Sigma). These preparations were frozen by submersing in liquid nitrogen. The coverslips were removed, and slides were stored in 95% ethanol until they were immunostained.

HSF Immunolocalization on Polytene Chromosomes—Salivary gland chromosome preparations were stained for HSF as described previously (22), with a primary rabbit anti-HSF antibody (943) and a secondary fluoroscein isothiocyanate-conjugated goat anti-rabbit antibody (Cappel, catalog no. 55655). DNA was stained for fluorescence with Hoechst 33342 (Sigma). Photography was performed using a Nikon Microphot fluorescence microscope, a Nikon Plan 40× objective and Fujichrome Sensia 400 ISO film. Exposure times ranged from 2 to 6 s (with neutral density filters) for Hoechst-stained preparations, and from 25 to 40 s for fluoroscein isothiocyanate-stained samples. 35-mm transparencies and autoradiograms were digitized using an Agfa Arcus II scanner. When necessary, digitized images were adjusted for contrast and brightness using Adobe Photoshop.

Tissue Culture—Drosophila Schneider line 2 (SL2) cells were used for all experiments involving tissue culture cells. Cells were grown in Shields and Sang M3 insect medium (Quality Biological, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and 20 μg/ml gentamycin solution (Sigma). Cells were grown at 21–23 °C in T-75 tissue culture flasks (for suspension cells; Starstedt). Prior to all experiments, cells at a concentration of 1–1.4 × 10^7 cells/ml were transferred to 50-ml polypropylene tubes at a volume of 2.5–6.5 ml. Cells were aerated in these tubes by shaking at 175 rpm, 21 °C, for 4 or more h prior to the experiment.

Cell Treatments—21 °C controls were prepared by taking cells that had not been subjected to any stress agents, but had undergone the aeration described above. All other cell treatments were also performed after aeration. 36.5 °C heat shocks were performed by incubating the cells in the polypropylene tubes in a temperature-controlled circulating water bath (Neslab). Salicylate was added to cells from a 1 M sodium salicylate stock prepared in a physiological *Drosophila* saline (45 mM potassium glutamate, 45 mM sodium glutamate, 8.7 mM $MgSO_4$, 5.0 mM Bis-Tris, 6.8 mM $CaCl_2 \cdot H_2O$, 12 g/liter glucose, pH 6.9), to the concentrations indicated in each experiment. 30 mM salicylate plus heat shock experiments were performed by incubating cells in 30 mM salicylate for 5 min at 21 °C and then transferring the cells to 36.5 °C for the remaining time interval. For experiments studying the effects of protein synthesis inhibition, cycloheximide was added to cells at a concentration of 118 μM 30 min prior to further cell treatments (23). Cells were continually shaken at 175 rpm during this period.

Protein Extracts for Mobility Shift Assays—Cells were treated to the conditions described in each experiment. 1-ml aliquots of cells were transferred to 1.5-ml microcentrifuge tubes, and pelleted at 7000 rpm, at 4 °C, for 2 min in a Beckman microcentrifuge. Supernatants were aspirated off, and cell pellets were immediately frozen under liquid nitrogen and either stored at −72 °C or processed immediately as follows: pellets were thawed by resuspension in five pellet volumes of lysis buffer (10 mM HEPES pH 7.9, 0.4 M NaCl, 0.1 mM EGTA, 5.0% glycerol, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and then centrifuged at 10,000 × *g* for 10 min at 4 °C. Supernatants were then transferred to a new 1.5-ml tube, frozen under liquid nitrogen, and stored at −72 °C.

EMSA—An HSE consensus sequence (HSE3; 5′-GGG CGT CAT AGA ATA TTC TCG AAT TCT GGG TCA GG-3′) was annealed to a shorter complimentary oligonucleotide (5′-CC TGA CCC AGA ATT CGA G-3′) and the overhang was filled in using the Klenow fragment of DNA polymerase with 0.167 mM each of dATP, dTTP, and dGTP; 50 μCi of [α-^{32}P]CTP (3000 Ci/mmol; DuPont NEN); and 2.5 units of Klenow (New England Biolabs) used in the labeling reaction. Unincorporated nucleotides were removed using a gel filtration (Sephadex G25, Pharmacia) spin column. Gel shift assays were performed essentially as described in Ref. 16. 4 μl of *Drosophila* SL2 cell extract was mixed with 6.1 μl of reaction mix containing: 4 μl of ddH_2O, 1 μl of 10 × buffer mix (100 mM HEPES, pH 7.9, 500 mM NaCl, 30% glycerol, w/v), 1 μl of 10 × bovine serum albumin/nucleotide mix (0.5 mg/ml *Escherichia coli* DNA, 0.2 mg/ml poly-d$(N)_6$, 2 mg/ml yeast tRNA, 20 mg/ml bovine serum albumin), and 0.1 μl of ^{32}P-labeled HSE3 (0.01 pmol). The binding reaction was allowed to proceed for 10 min on ice (0 °C) and then 2 μl of 6 × loading dye (0.25% bromphenol blue, 0.25% xylene cyanole, 30% glycerol (w/v), 3 × TBE) was added. Samples were electrophoresed in a 1% agarose gel (Seakem ME) for 1.25 h at 82 V in 0.5 × TBE buffer. Gels were blotted and dried onto Whatman DE81 paper and exposed to preflashed Kodak XRP-1 film with a Cronex Lightning Plus intensifying screen (DuPont) at −72 °C.

RNA Preparation—For SL2 cells, 5 × 10^7 cells were used for each preparation. After treatment, cells were pelleted and washed once in one volume of *Drosophila* saline. RNA extractions were performed using the guanidinium/CsCl method for total RNA extraction described in Ref. 24. For salivary glands, total RNA was extracted from 20 pairs of salivary glands for each treatment studied and glands were treated in the same manner as for immunostaining. RNA was prepared using the RNeasy RNA extraction kit (Qiagen). RNA was quantified by spectrophotometry. RNA samples were stored at −72 °C until use in primer extension analysis.

Primer Extension Assays—For SL2 cells, 5 or 10 μg of total RNA was lyophilized and resuspended in an 18.5-μl final volume of buffer containing 50 mM Tris-Cl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 20 units/μl RNAguard (Pharmacia), and 0.1 pmol of ^{32}P-end-labeled hsp 70 oligomer (5′-CCC AGA TCG ATT CCA ATA GCA GGC-3′) and/or H2B oligomer (5′-GCC TTT CCA CTA GTT TTC GGA GGC-3′). For salivary glands, 1 or 2 μg of total RNA was lyophilized and resuspended in 9.25 μl of the above buffer and 0.05 pmol of each oligomer was added. Oligomers (10 pmol) were end labeled using 30 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, DuPont NEN) and T4 polynucleotide kinase (New England Biolabs) according to Ref. 24, followed by removal of unincorporated ATP with a Sephadex G-25 (Pharmacia) spin column. The reaction was heated to 85 °C for 5 min and then transferred to 42 °C and incubated overnight (14–18 h). The next morning dNTPs were added to a final concentration of 2 mM, and then 100 units of reverse transcriptase (Superscript II; Life Technologies, Inc.) to give a final total volume of 20 μl (or 50 units of reverse transcriptase and a final volume of 10 μl for salivary gland RNA). The reaction was then incubated at 42 °C for another 60 min. Primer extension reactions were then ethanol-precipitated with 3 μl of 2 M sodium acetate and 50 μl of 100% ethanol, washed with 70% ethanol, and dried. Primer extension products were resuspended in 10 μl of formamide loading dye (Stop solution, U. S. Biochemical Sequenase Rapidwell DNA sequencing kit), denatured by heating to 100 °C, and electrophoresed on an 8% PAGE, 7 M urea gel in 1 × TBE, which had been prerun for 30 min at 200 V. Gels were run until the bromphenol blue marker had run 2/3 of the length of the gel. The gel was dried and autoradiographed using Kodak XAR5 film at −72 °C with a Lightning Plus (DuPont) intensifying screen. The

molecular sizes of the hsp 70 and H2B primer extension products were originally determined on large gels (35 × 40 cm) and determined to be 275 bp (for hsp 70) and 78 bp (for H2B), which was in good agreement with the sizes of 270 and 70 bp estimated from the sequences of the genes.

Densitometry of Autoradiograms—All autoradiograms that were to be analyzed by densitometry were prepared on Kodak XRP-1 film, which had been preflashed with an Amersham Sensitize flash unit to obtain a linear range of exposure. Autoradiograms were digitized using an Agfa Arcus II scanner. Densitometric analysis was performed using the profile analyst feature of the Molecular Analyst software program (Bio-Rad). For the EMSA assays, HSF binding activity was expressed as a percentage of the heat shock (36.5 °C) cell sample. For the primer extension assays, the hsp 70 signal intensities were first normalized using the H2B signals. These normalized values were then used to compare signal intensities between different treatments and the results were expressed as the ratio (fold) of hsp 70 message relative to the control (21 °C) level. In some instances no hsp 70 signal was detectable under control conditions, and thus relative amounts could not be calculated.

ATP Assays—Intracellular ATP was measured using Boehringer Mannheim's Constant Light Source Kit II. Cell extracts were prepared as per the instructions included with the kit. 110 μl of cells (approximately 1×10^6 cells) were added to 1000 μl of boiling 100 mM Tris, pH 7.75, 4 mM EDTA solution. Samples were boiled for 2 min and then centrifuged for 3 min at 10,000 × *g* and 4 °C. Supernatants were transferred to a clean microcentrifuge tube and kept at 4 °C. For each reading, 100 μl of the extract was added to 100 μl of the luciferase reagent provided in the kit. Readings were taken using a scintillation counter (Wallac model 1409) over 20-s intervals with the windows wide open. All conditions were performed in triplicate. ATP levels were determined with the aid of a standard curve prepared using pure ATP over a 10^{-5} to 10^{-9} M concentration range.

Protein Labeling and Immunoprecipitation—100 μCi of $^{32}P_o$ phosphate (DuPont NEN) was added to 1 ml of cells at 1.0×10^7 cells/ml in phosphate-free M3 medium. Immediately after addition of phosphate, salicylate was added to the cells, or cells were heat-shocked by submersion in a 36.5 °C water bath. After the appropriate time interval, cells were pelleted by centrifugation at 10,000 × *g* for 30 s. Supernatants were removed, and 10 volumes of TETN400 buffer was added (25 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 7.5), 400 mM NaCl, 1% Triton X-100 v/v) (25). Pellets were immediately lysed and resuspended by drawing through a 28.5-gauge needle. Samples were stored at −72 °C until processed by immunoprecipitation. Immunoprecipitations were performed as described in Ref. 25 with a few modifications. Cell extracts were made using a buffer with increased salt concentration (400 mM NaCl) and thus in order to maintain a 250 mM NaCl concentration, an equal volume of TETN100 (5 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 7.5), 100 mM NaCl, 1% Triton X-100 v/v) rather than TETN250 was added to each extract for binding reactions to give a final 250 mM NaCl concentration. 1 μl of anti-*Drosophila* HSF antiserum (943) (22) was added to each 100-μl binding reaction, and the entire sample was immunoprecipitated and loaded onto a 8% PAGE gel.

RESULTS

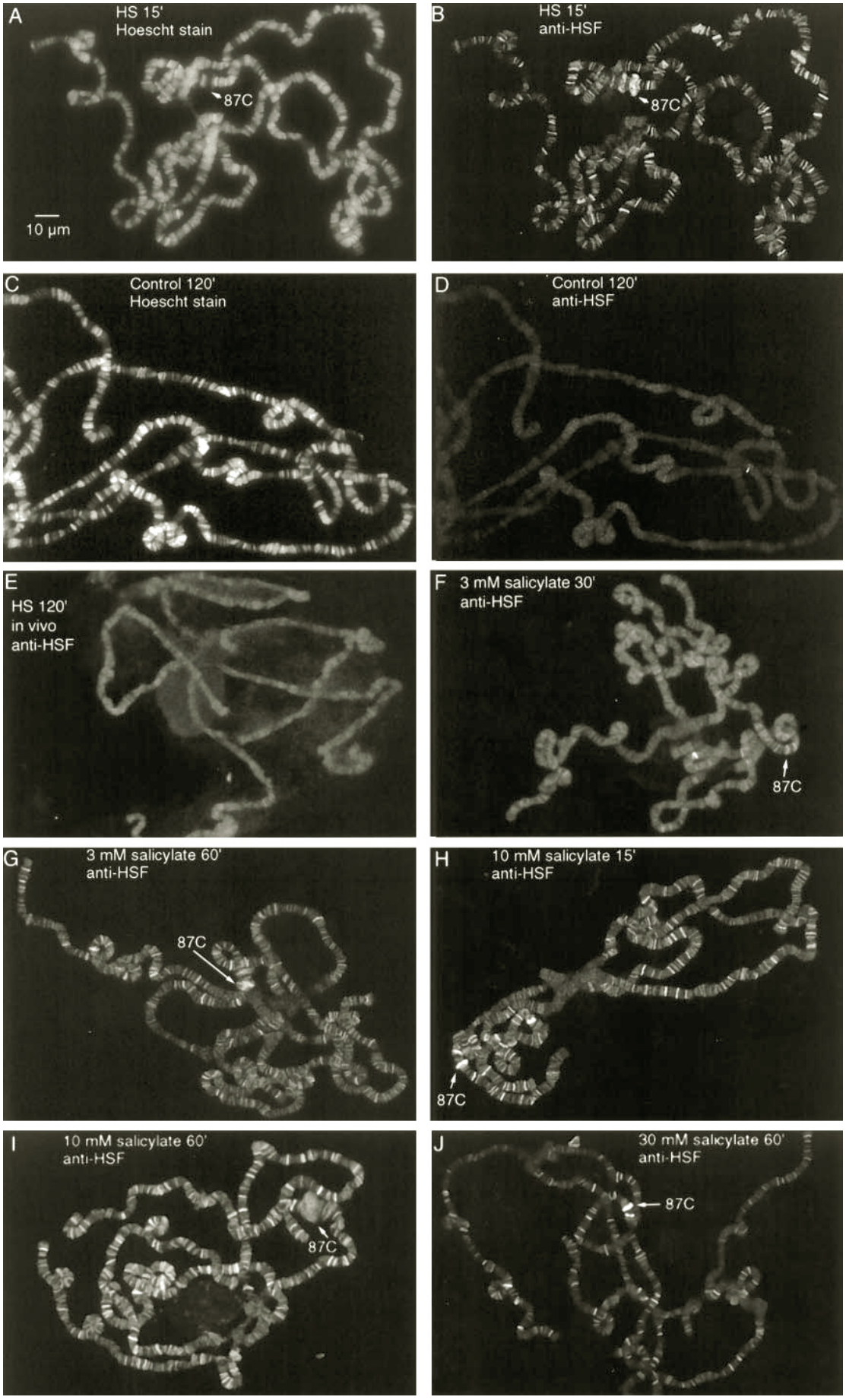
Salicylate Induces HSF Binding and Chromosomal Puffing at Heat Shock Gene Loci in Polytene Chromosomes of D. melanogaster Salivary Glands—HSF has been shown previously to undergo a change in its DNA binding pattern on the polytene chromosomes of *D. melanogaster* third instar salivary gland cells when the larvae are exposed to heat stress (22). Glands dissected at room temperature showed a diffuse, nonspecific association of presumably inactive HSF with the polytene chromosomes (Fig. 1*D*). Upon an increase in temperature (for instance, 15 min at 36.5 °C), HSF is found localized at numerous specific chromosomal sites (Fig. 1*B*). Most notably, it is bound to all nine of the heat shock gene sites and has induced the corresponding heat shock puffs. These sites include the hsp 70 gene loci; two copies of hsp 70 are found at 87A and three copies at 87C. In this study we have mostly limited our description on puff sites to the 87C locus, the largest and easiest to identify of the heat shock puffs. It was observed in the present series of experiments that the previously published *in vivo* (whole larvae) findings could be duplicated in an *in vitro* (isolated salivary glands) heat shock assay (Fig. 1*B*). The ability to induce the heat shock response in isolated salivary glands maintained in organ culture allowed for the easy addition of chemical agents. One set of conditions where *in vivo* and *in vitro* results did differ was during a prolonged heat shock. A prolonged (60–120 min) *in vivo* heat shock, resulted in HSF staining returning to a non-shock type staining pattern (Fig. 1*E*), whereas a prolonged *in vitro* heat shock resulted in a partial degradation of the salivary gland preparations that proved to be refractory to staining (results not shown). Control (room temperature) glands did not appear to degrade and stained normally during a prolonged incubation *in vitro* (at least up to 180 min) (see Fig. 1, *C* and *D*).

The addition of sodium salicylate yielded a temporally different HSF binding pattern and a temporal and qualitatively different heat shock puffing pattern than heat stress. At a concentration of 3 mM, salicylate was found to be able to induce both HSF binding and heat shock puffing in the salivary glands. Within 30 min, binding was consistently evident at high affinity heat shock gene sites (such as the 63BC chromosomal locus, the site of the hsp 82 gene and 87A and 87C) (Fig. 1*F*). Binding at all of the heat shock puff sites, however, was not observed. By 60 min of induction, however, binding patterns were comparable to those seen in a 15-min heat shock (Fig. 1*G*). Puffing was also observed at the heat shock loci, although visual examination of the 87C locus suggested that the degree of puffing was noticeably less than that seen in heat shock. Continued exposure past 60 min saw an apparent partial recovery from the response and by 120 min, HSF was bound in considerably lesser amounts, and puffing at the heat shock loci appeared to be diminished (data not shown). Collectively, these results suggest that a mild response to salicylate occurs at the 3 mM concentration. While HSF activation and heat shock puffing does occur, it does so over a longer time scale and both the intensity of HSF staining and the size of the puffs are less than what a heat shock produces.

By comparison, a rapid response was observed with a 10 mM salicylate treatment. The activation and the binding of HSF was observed within 2 min and, similar to heat shock, full binding was evident within 5 to 15 min. While a small degree of puffing was detectable at 87C within 15 min of induction (Fig. 1*H*), maximal levels were not obtained until 45–60 min of continuous salicylate treatment, and at this point, the degree of puffing and the sites that are puffed (Fig. 1*I*, but only the 87C puff is identified) was comparable to a 15-min heat shock (Fig. 1*B*). Thus, the binding of HSF and the activation of heat shock puffing in 10 mM salicylate displays separate kinetics. No recovery of the response was observed at 180 min after induction; full levels of HSF binding and heat shock puffing were still present (data not shown). This differs to what is observed during an *in vivo* heat shock where HSF binding and heat shock puffs return to an unshocked pattern by 60–120 min (Fig. 1*E*).

A 30 mM salicylate treatment also causes rapid HSF binding kinetics. Within 1–2 min, considerable levels of HSF binding were observed, with full levels apparent after about 5 min (data not shown). Very small or no puffs were detected within 5–15 min of induction, and no further increases in puff size were recorded (Figs. 1*J* and 2*A*). As in the case with 10 mM salicylate, recovery from the response was not observed. Similar to a prolonged heat shock, prolonged (60–120 min) exposure to 30 mM salicylate resulted in the partial degradation of the gland and poor staining (data not shown).

At optimum time points, all of the concentrations tested displayed similar HSF staining intensities. Although the 30 mM condition appeared to stain brighter, this was likely due to


A
HS 15'
Hoescht stain
87C
10 μm
B
HS 15'
anti-HSF
87C
C
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D
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HS 120'
in vivo
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10 mM salicylate 15'
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10 mM salicylate 60'
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30 mM salicylate 60'
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FIG. 1

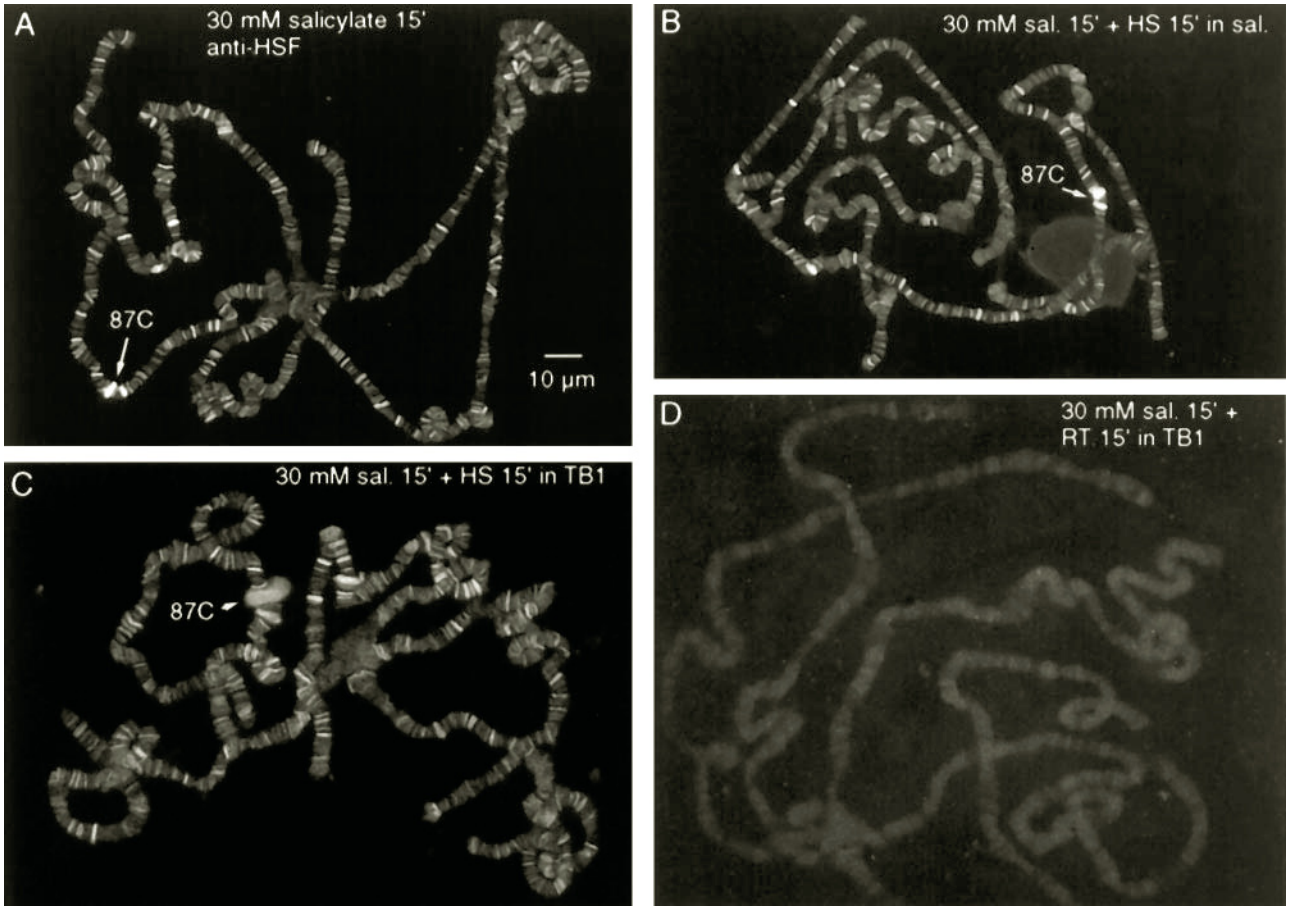


FIG. 2. **30 mM sodium salicylate prevents induction of the heat shock puffs by heat.** Salivary glands were dissected as described in Fig. 1 and chromosomal squashes prepared from glands treated with 30 mM salicylate (*A*), 30 mM salicylate plus a heat shock (15 min 36.5 °C) in the presence of salicylate (*B*), 30 mM salicylate followed by a heat shock in the absence of salicylate (*C*), and 30 mM salicylate followed by an incubation in normal buffer at the control temperature (*D*). HSF was stained as described in Fig. 1. The *bar* represents 10 μm.

the absence of puffs causing a condensation, and thus a concentration, of intensity in the staining region.

To investigate the possibility that high amounts of salicylate might be inhibitory toward heat shock gene transcription, glands were incubated first for 15 min in 30 mM salicylate and were then heat-shocked in the presence of salicylate. The results of this experiment show that heat shock-induced puffing is indeed prevented in the presence of 30 mM salicylate (Fig. 2*B*). In a similar experiment, glands were treated for 15 min in 30 mM salicylate and then heat-shocked in the absence of salicylate. Under these conditions, full HSF binding and puffing patterns were observed (Fig. 2*C*). These results suggested that high concentrations of salicylate inhibited heat shock puffs and that the inhibitory effect of salicylate on puffing was reversible when it was removed. A summary of salicylate-induced HSF binding and puffing at the 87C locus is shown in Table I.

Salicylate Induces the Binding of HSF to DNA in Drosophila Tissue Culture Cells—In SL2 cells, HSF binding activity to an HSE was found to be induced by salicylate in a dose-dependent manner (Fig. 3*A*). Binding of HSF was activated within 20 min at 3, 10, and 30 mM concentrations of salicylate. Quantification of the autoradiogram using densitometry revealed that these three salicylate concentrations induced HSF binding activity to 33, 75, and 80%, respectively, of the activity observed with a 36.5 °C heat shock. The HSF binding pattern for heat and salicylate-treated cells at 60 min was similar to the 20 min pattern (data not shown). When cells were heat-shocked (36.5 °C) in the presence of 30 mM salicylate, the amount of HSF binding activity was greater (102% of the 36.5 °C sample) than 30 mM salicylate alone (80% of the 36.5 °C sample) (Fig. 3*A*). When cells were exposed to 30 mM salicylate for 20 min, washed once in saline, and resuspended in salicylate-free media for 40 min, the salicylate-induced DNA binding of HSF was reversed (Fig. 3*A*). The level of HSF binding in cells treated in such a manner was reduced to levels comparable to a normal 21 °C control (20 and 15%, respectively, of the 36.5 °C sample). If cells treated in a similar fashion were heat-shocked after resuspension in salicylate-free media, a typical heat shock-induced level of HSF binding was observed (91% of the 36.5 °C sample) (Fig. 3*A*). Heat shock was capable of triggering HSF binding in the presence of the protein synthesis inhibitor cycloheximide, and cycloheximide by itself did not induce HSF binding within the 50-min time interval of the experiment (Fig. 3*B*). Cycloheximide treatment modestly affected HSF binding

FIG. 1. **Sodium salicylate induces HSF binding and heat shock puffs in the polytene chromosomes of *Drosophila* third instar salivary glands.** Salivary glands from *D. melanogaster* third instar larvae were dissected in TB1 buffer and kept in organ culture for a minimum of 60 min prior to stress treatment. Chromosomal squashes were prepared from control glands (21 °C, *C* and *D*), heat-shocked glands (15 min 36.5 °C, *A* and *B*), or glands treated with 3, 10, or 30 mM sodium salicylate (*E–J*). The duration of exposure to salicylate is indicated. Chromosomes were stained for DNA using bisbenzimide (Hoescht) (*A* and *C*) and for HSF using a rabbit anti-HSF primary antisera (943) followed by a goat anti-rabbit fluoroscein isothiocyante labeled secondary antibody (*B*, *D–I*). *87C* is the location of three copies of the hsp 70 gene. The *bar* represents 10 μm.

TABLE I

Summary of HSF staining and chromosomal puffing at the 87C (hsp 70) locus

The degree of HSF staining and HS puffing at the 87C locus in response to the conditions indicated was estimated by visual examination under fluorescence microscopy. Scores ranged from + (lowest degree) to +++++ (highest degree) of staining or puffing; − reflects no staining or puffing. These rankings are given at the indicated time points and are when the staining or puffing appeared to be maximal.

Condition	HSF staining intensity (87C)		HS puffing (87C)	
	Optimal time	Degree	Optimal time	Degree
Room temperature (21 °C)		−	−	−
Heat shock (36.5 °C)	5–15 min	++++	15 min	+++++
3 mM salicylate	60 min	++++	60 min	++½
10 mM salicylate	5–15 min	++++	45–60 min	++++½
30 mM salicylate	5 min	+++++	5–15 min	+/−
30 mM salicylate + HS	15 min salicylate + 15 min HS in salicylate[a]	+++++	15 min salicylate + 15 min HS in salicylate[a]	+/−

[a] This was the only time point performed for these conditions.

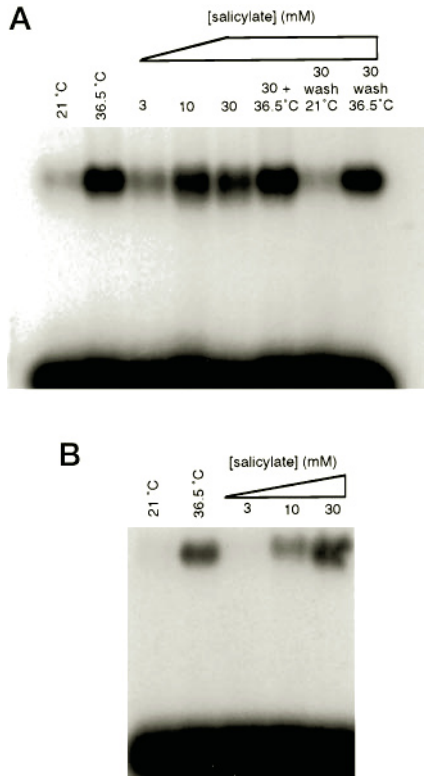


FIG. 3. **Sodium salicylate activates the binding of HSF to HSEs in *Drosophila* SL2 cells in the absence of protein synthesis.** *A*, *Drosophila* tissue culture cells at a concentration of 1×10^7 cells/ml were exposed to the conditions indicated for a period of 20 min. Cell extracts were prepared and HSF binding activity was analyzed by electrophoretic mobility shift assays. A radiolabeled HSE consensus sequence was used for a probe. *B*, the experiment was performed as described in *A* with the exception that prior to each condition, cells were incubated with 118 μM cycloheximide for 30 min in order to prevent protein synthesis (23).

induced by 10 or 30 mM salicylate treatments (51 and 116% binding activity, respectively, when compared to the 36.5 °C cycloheximide-treated sample) (Fig. 3*B*). HSF binding in the control (21.5 °C) sample and in the 3 mM salicylate-treated cells appeared to be inhibited by cycloheximide, as both had 1% of the HSF binding activity of the 36.5 °C cycloheximide-treated sample. The ability of cycloheximide to inhibit induction of HSF binding has previously been observed in *Drosophila* for control and submaximal heat shock temperatures (23). These results suggest that protein synthesis is not a prerequisite for induction of HSF binding activity by higher concentrations of salicylate (10 and 30 mM) but may play a role in the induction of HSF binding activation at lower salicylate concentrations (3 mM). This finding differs somewhat from one reported in mammalian cells where cycloheximide treatment prevented induction of HSF binding by salicylate at all of the concentrations tested (18).

Salicylate Fails to Induce the Transcription of hsp 70 in Drosophila Salivary Glands and SL2 cells—In an attempt to correlate the induced puffing of the 87C locus of the polytene chromosomes with transcription of the hsp 70 gene, primer extension analysis was performed to assay the levels of hsp 70 message present in the salivary glands (Fig. 4). Little detectable message was present at the control temperature (21 °C). Upon heat shock (36.5 °C for 20 min), the amount of hsp 70 message detected greatly increased (approximately 50-fold as determined by densitometry) (Fig. 4) and this correlated with large puffs at 87C and 87A (Fig. 1*B*). A 10 mM salicylate treatment for 1 h induces maximal puffing at the hsp 70 loci (Fig. 1*G*), but no hsp 70 message could be detected by primer extension analysis (Fig. 4). This result was surprising, given that heat-induced puffing at the heat shock gene loci has previously been correlated with heat shock gene transcription (see "Discussion"). This result also suggests that chromosomal puffing and transcription of the heat shock gene loci can be uncoupled.

To ascertain the effects of heat and salicylate treatments on heat shock gene expression in SL2 cells, hsp 70 mRNA levels were also monitored by primer extension analysis. During heat shock (36.5 °C) the amount of hsp 70 transcripts increased approximately 130–150-fold with large amounts of hsp 70 extension products seen at 20 min (Fig. 5*A*) and at 60 min (Fig. 5*B*). None of the concentrations of salicylate used (3–30 mM) were capable of inducing hsp 70 expression within the 60-min interval. Furthermore, 30 mM salicylate prevented heat-induced expression of the hsp 70 genes. Washing the cells and allowing for recovery did not cause an increase in hsp 70 transcripts. However, after a recovery period, heat shock inducibility of hsp 70 gene expression was restored (Fig. 5*C*).

These results give an indication of steady state hsp 70 message levels in cells and do not rule out the possibility that hsp 70 messages might be induced but are extremely unstable in salicylate-treated cells. Methods can be used to detect newly initiated transcripts (*e.g.* nuclear run-on analysis). However, most of these methods require a subsequent *in vitro* step utilizing excess ATP, and because salicylate affects ATP levels (see next section), these types of analyses are less likely to give an accurate picture of hsp gene expression in salicylate-treated cells.

Salicylate Causes a Rapid Decrease in Intracellular ATP Levels in Drosophila SL2 Cells—While investigating the induction of HSF binding activity by a number of different inducers of the heat shock response, we noticed that induction caused by sodium salicylate was at least temporally very similar to that

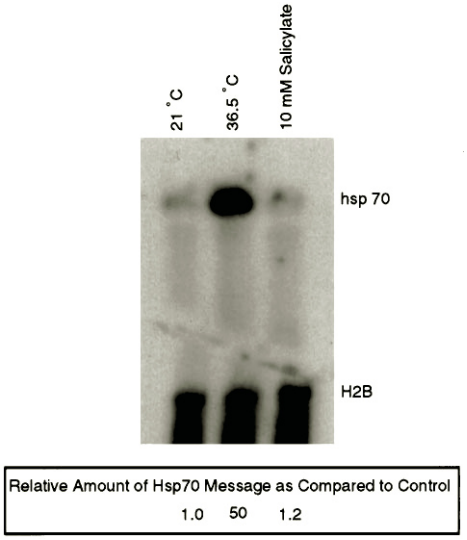
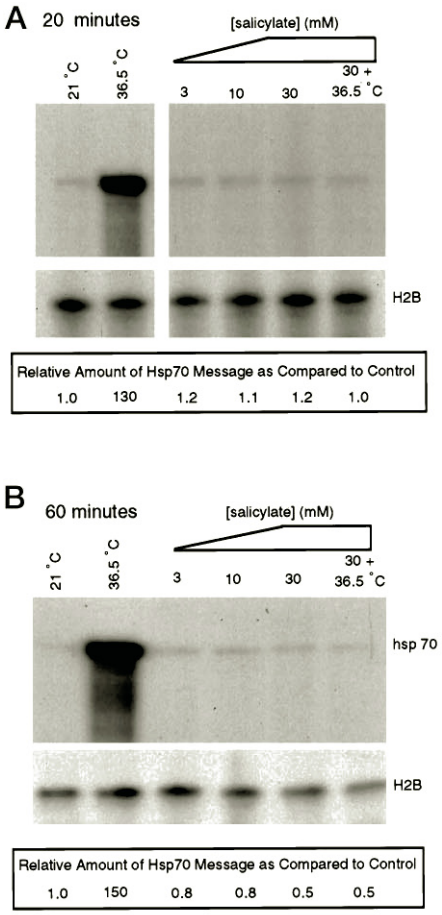


FIG. 4. **Sodium salicylate does not activate hsp 70 gene transcription in *Drosophila* salivary glands.** RNA extracts were prepared from *Drosophila* salivary glands, which were incubated for 2 h in TB1 buffer at 21 °C, incubated 1.5 h at 21 °C in TB1 and then heat-shocked at 36.5 °C for 20 min, or incubated 1 h at 21 °C in TB1 then for 1 h in 10 mM sodium salicylate. Heat-shocked and salicylate-treated salivary glands show maximal puffs at the 87C locus at these time points. Hsp 70 and histone H2B gene transcripts in these RNA samples were identified by primer extension analysis. Densitometry was performed with the aid of the Molecular Analyst program (Bio-Rad) (see "Experimental Procedures"). Hsp 70 message signals were first normalized by comparing them to the H2B message signals. After normalization, the ratio of hsp 70 message in comparison to the level present in the control (21 °C) sample was calculated and is shown below the autoradiogram.



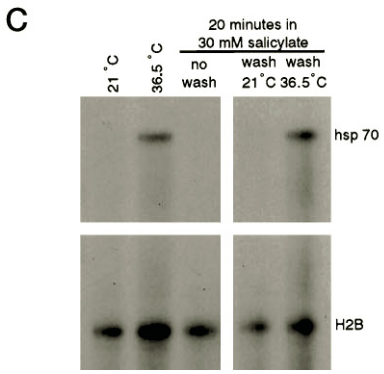


FIG. 5. **Salicylate fails to induce hsp 70 gene transcription in SL2 cells and inhibits heat-induced hsp 70 transcription.** Total RNA extracts were prepared from *Drosophila* SL2 cells after exposure to the noted conditions for 20 min (*A*) or 60 min (*B*). The amounts of hsp 70 and H2B gene transcripts were determined by primer extension analysis. *C*, the experiment was performed as described above using extracts from cells which were treated for 20 min with 30 mM salicylate, washed in saline, and allowed to recover 20 min at 21 °C. Cells were then either kept at 21 °C or heat-shocked at 36.5 °C for an additional 20 min. Densitometry was performed as described in the legend of Fig. 4. No values are given for *C*, as the signals present in the control (21 °C) sample were not strong enough to be detected, and thus ratios to this value could not be made.

seen by induction by 2,4-dinitrophenol and other inhibitors of oxidative respiration.[2] This prompted us to examine what effect sodium salicylate might have on ATP production inside of *Drosophila* cells. Under normal physiological conditions, the amount of intracellular ATP in *Drosophila* SL2 cells was found to remain fairly constant (results not shown). Heat shock reduced intracellular ATP levels by approximately 35% (Fig. 6). Salicylate treatment had a rapid and dramatic effect on the level of ATP within the cells. 3 mM salicylate dropped ATP levels slightly below that seen in heat shock (*i.e.* a 40–45% reduction), and higher concentrations of salicylate decreased this level even further. 10 mM salicylate decreased ATP levels by more than 60%, and 30 mM salicylate by more than 75%. The majority of this effect was seen within 5 min of the addition of salicylate. Heat shocking cells in the presence of 30 mM salicylate had the greatest effect on intracellular ATP where levels dropped by more than 85% when compared to untreated cells.

Salicylate Does Not Induce the Hyperphosphorylation of HSF—Heat shock has been reported to induce the hyperphosphorylation of HSF in yeast and human cells (26, 27), and it has been suggested that this modification could play a role in HSF activation and/or inactivation (see "Discussion"). The phosphorylation of *Drosophila* HSF (dHSF) in response to heat and salicylate treatments was assayed by immunoprecipitation of HSF from SL2 cells transiently labeled with [^{32}P]orthophosphate. Heat shock induced the hyperphosphorylation of dHSF (Fig. 7). Salicylate treatments, however, did not induce this hyperphosphorylation of HSF. In fact, salicylate treatments decreased the amount of HSF phosphorylation normally seen in untreated cells (Fig. 7, compare 21 °C to salicylate treatments). At 10 and 30 mM salicylate, no phosphorylation of HSF is detected at all. Surprisingly, 30 mM salicylate also prevented the heat-induced hyperphosphorylation of HSF.

DISCUSSION

Sodium salicylate has long been known to be an inducer of heat shock puffs in *Drosophila* salivary glands (5, 6). These

[2] N. A. Winegarden and J. T. Westwood, unpublished observations.

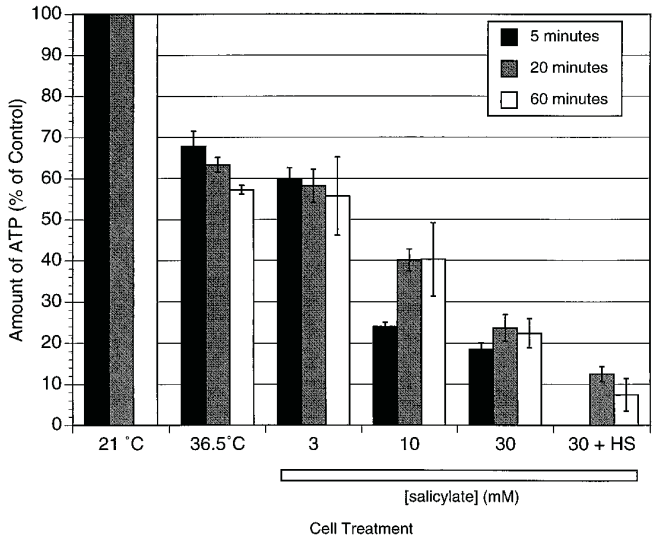


FIG. 6. **Salicylate rapidly decreases intracellular ATP levels in a dose-dependent manner.** *Drosophila* SL2 cells were subjected to each of the treatments indicated. Cells were lysed and proteins denatured by boiling in 100 mM Tris (pH 7.75), 4 mM EDTA solution for 2 min. ATP levels were determined with the use of a luciferase based assay and detected with a scintillation counter. Values are expressed as percent of control (21 °C), which was approximately 2.7×10^{-4} mM ATP/10^5 cells.

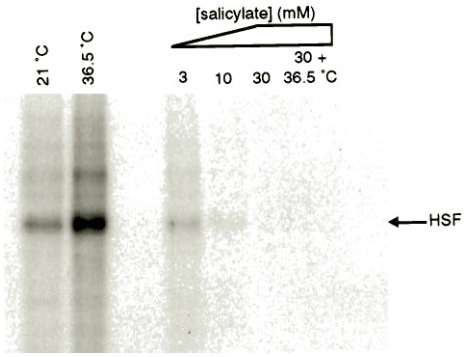


FIG. 7. **Salicylate inhibits the phosphorylation of *Drosophila* HSF.** HSF was immunoprecipitated from extracts of *Drosophila* SL2 cells, which were treated with the conditions indicated for 20 min, in the presence of ^{32}P-labeled *o*-phosphate. Immunoprecipitates were separated on 8% SDS-PAGE gels and autoradiograms were made. The band representing HSF is indicated.

puffs have been assumed to be the sites of heat shock gene transcription and, for heat-induced puffing, this has been demonstrated to be true (for review, see Ref. 8). More recent studies in human cells (17, 18) and in *S. cerevisiae* (19) have suggested that sodium salicylate can induce certain but not all aspects of the heat shock response. That is, salicylate induces HSF binding at the promoters of heat shock genes, as demonstrated by *in vivo* footprinting. However, salicylate's ability to activate heat shock gene transcription appears to be dependent on the concentration of salicylate used in human cells (17) and does not occur at all in yeast (19). These observations prompted us to reexamine the salicylate-induced heat shock response in *Drosophila* using both salivary glands from third instar larvae and Schneider line 2 (SL2) cells.

The results of this study show that salicylate (3–30 mM) induces HSF binding activity and that binding precedes puffing of the heat shock gene loci in the salivary gland polytene chromosomes of third instar larvae. With concentrations above 10 mM, HSF binding occurred with kinetics similar to those seen with a heat shock. At 3 mM, however, full binding of HSF occurred on a much slower time scale. In terms of puffing, and what we initially assumed to be transcriptional activation of the heat shock genes, the salivary gland chromosomes displayed varying responses to different salicylate concentrations. At the 3 mM concentration, optimal puffing occurred at the same time as optimal HSF binding, about 60 min into induction. The degree of puffing was not as high as that seen in heat shock, suggesting overall that the response to 3 mM salicylate was relatively mild. At 10 mM salicylate, maximal puffs, similar in size to those seen in a heat-induced response, were observed. However, maximal puffing did not occur until well after maximal HSF binding occurred with this difference in kinetics suggesting that HSF binding and puff induction are separable events. At 30 mM salicylate, puffing appeared to be inhibited. Experiments in which heat shock was used in conjunction with 30 mM salicylate indicated that high concentrations of this agent inhibited heat-induced puffing. In summary, the kinetics and the dynamics of HSF binding and puffing in response to salicylate suggest that these two processes are separable.

Salicylate inhibits ATP concentrations in *Drosophila* SL2 cells in a dose-dependent manner. Heat shock also decreases intracellular ATP but not to the same extent as the salicylate concentrations used in this experiment. 3 mM salicylate decreases ATP to levels slightly below what heat shock does (40% *versus* 35% inhibition) and weakly activates HSF binding. Higher concentrations of salicylate dramatically affect cellular ATP; 10 mM salicylate decreases ATP by up to 60% and 30 mM by up to 75% of control levels. Salicylate has previously been reported to decrease intracellular ATP in rat astrocytes (28). In SL2 cells, most of this inhibitory effect is exhibited within 5 min of the addition of salicylate. This time course of ATP inhibition correlates well with the activation of HSF binding by salicylate indicating that decreases in intracellular ATP may be an important factor in the activation of HSF binding by this drug.

Numerous inducers of heat shock puffs in *Drosophila* have been shown to be inhibitors of oxidative respiration (see Refs. 8 and 29–32). The concept that decreased cellular ATP levels could play an important role in the activation of heat shock puffs in *Drosophila* was suggested in a number of early studies (7, 29, 33). There was good evidence showing that heat and certain inhibitors of oxidative respiration indeed did lower intracellular ATP levels and induced puffing at the heat shock gene loci (31, 32). However, the authors of these studies concluded that decreased cellular ATP was not the causative agent of induction chiefly based on experiments in which certain inhibitors or combinations of inhibitors lowered cellular ATP but did not induce puffing. Our results show that high concentrations of oxidative respiration inhibitors (*e.g.* 30 mM salicylate) induce HSF binding with no or minimal puffing, suggesting that such inhibitors can activate the heat shock response to varying degrees. We propose that in *Drosophila*, all agents that lower intracellular ATP will likely activate HSF binding.

Sodium salicylate does not appear to induce heat shock gene transcription, even at the concentrations (3 and 10 mM) that clearly induced heat shock puffs. This was a surprising result, given that previous studies had demonstrated that radiolabeled RNA precursors are incorporated into heat-induced heat shock puffs and that inhibitors of RNA synthesis such as actinomycin D and α-amanitin block the formation of the heat shock puffs (Refs. 7 and 34; see Ref. 8 for a review). We interpret this result to signify that for heat shock genes, puffing of the gene(s) does not necessarily mean the gene is actively transcribing.

What does puffing represent? Studies on the intermolt puff found at 68C, the site of three salivary gland secretion (sgs) protein or glue genes, have led Meyerowitz and co-workers to

question whether puffs actually signify transcription. The insertion of the three 68C glue genes, *Sgs-3, Sgs-7 and Sgs-8*, into the germline of *Drosophila* does produce a new puff at the insertion site (35). Likewise, new heat-inducible puffs have been reported by Lis and colleagues in *Drosophila* having either a hsp 70 gene with its promoter or a hsp 70 gene promoter fused to the *lacZ* gene inserted into their germline (36, 37). However, transformants that contain only the *Sgs-3* gene do not have puffs at the insertion sites even though the inserted *Sgs-3* gene is transcribed at high levels (38). Furthermore, the reverse has also been demonstrated in the *l(1)npr-1* mutant, *i.e.* no glue gene mRNA is detected in this mutant even though the 68C locus is prominently puffed (39). Thus, Meyerowitz and co-workers have observed situations where highly transcribed genes do not produce puffs and puffs at sites which are not making mRNA. For salicylate-induced puffs, the reduction in ATP levels caused by salicylate might explain why puffing is observed but not transcription. Many of the enzymatic activities of RNA polymerase II holoenzyme, particularly those associated with the MO15, ERCC2, and ERCC3 subunits of TFIIH (40) have been shown to require ATP. ATP is also required as a substrate for the synthesis of the RNA molecule. Perhaps salicylate-induced puffs represent a localized decondensation of heat shock gene loci brought about by HSF binding and early transcription events such as formation of the transcription initiation complex and promoter clearance. However, the large size of the puffs induced by 10 mM salicylate would suggest that the decondensation of the polytene chromosome is likely occurring over a fairly large area (*i.e.* not just the promoter region). HSF binding alone does not appear to cause prominent puffs because 30 mM salicylate induced HSF binding but very small puffs or no puffs were observed. Further experiments will have to be performed to determine what puffs represent.

How does salicylate induce the high affinity binding of HSF to the HSE? It has been suggested that salicylate may be inducing HSF binding by generating abnormal proteins within the cell by a yet to be described mechanism (18). It has also been suggested that salicylate primarily affects newly synthesized proteins because the addition of cycloheximide prevented salicylate-induced HSF binding in human cells (18). In this study, we have found that in *Drosophila* cells, cycloheximide addition inhibited the induction of HSF binding by 3 mM salicylate but did not appear to inhibit the induction of HSF binding by 10 or 30 mM salicylate. Abnormal proteins have been proposed to be a "universal" intracellular stress inducer and are believed to be generated by a number of the agents which trigger the heat shock gene expression and heat shock protein synthesis (see Refs. 10 and 41–43). The newly synthesized heat shock proteins are then thought to go on to perform a number of functions including disaggregating, refolding and degrading denatured or abnormal proteins. Heat shock proteins (*e.g.* hsp/hsc 70) might also be directly responsible for maintaining HSF in its inactive configuration and/or converting the active DNA binding form of HSF back to its inactive form (see Refs. 13 and 14). Thus, the generation of abnormal proteins could substantially increase the number of substrates for hsc/hsp 70 and therefore, other substrates such as HSF may not be attended to, and in the case of HSF, "aggregate" to its active trimeric configuration.

Could reduced cellular ATP activate HSF binding? The hsp 70 family of heat shock proteins are ATPases that require hydrolyzable ATP to release bound peptides (44). Thus, if hsp 70 activity is required for maintenance of inactive HSF, any treatment that serves to substantially decrease ATP could result in the conversion of HSF to its high affinity DNA binding form. Alternatively, decreases in cellular ATP and the subsequent inhibition of heat shock proteins could lead to an accumulation of misfolded and partially folded proteins within the cell. An increase in abnormal proteins has been reported to occur in ATP-depleted cells (45).

Why is there no transcription of the heat shock genes in *Drosophila* salivary gland and SL2 cells even though HSF is binding to the HSEs? At present we cannot fully answer this question, but the results of this study suggest a number of possible explanations. In SL2 cells, ATP levels are reduced at all the concentrations tested (3–30 mM). The large reduction in ATP seen at 10 and 30 mM salicylate might account for the prevention of heat shock gene synthesis for the reasons described above, even when cells are heat-shocked. It would be difficult to invoke this hypothesis at 3 mM salicylate, given that ATP levels with this treatment are only reduced to approximately the same level as seen by a heat shock treatment. However, it should be noted that 3 mM salicylate only weakly induced HSF binding activity and, therefore, a large increase in heat shock gene synthesis might not be expected. Another possibility is that salicylate activated HSF is physically different from heat-activated HSF. All concentrations of salicylate used in this study reduced the amount of HSF that was phosphorylated and/or the number of phosphate groups per HSF molecule. In human cells, salicylate also appears to alter HSF phosphorylation (18). We observed that 30 mM salicylate prevented heat-induced heat shock gene transcription and the hyperphosphorylation of HSF that normally accompanies heat shock. These results indicate that HSF hyperphosphorylation is not required for the activation of HSF binding activity *in vivo.* A similar observation has been noted in human cells for certain stress agents (46). It has been proposed that hyperphosphorylation of HSF is a prerequisite for the transcriptional activation of heat shock genes in yeast and in human cells (26, 27, 47). If this hypothesis is true, the fact that salicylate treatments do not result in the hyperphosphorylation of HSF would explain why no transcription of the heat shock genes occurs in SL2 cells. However, the role of HSF hyperphosphorylation in the transcriptional activation of heat shock genes has been called into question, and in yeast, it appears that hyperphosphorylation of HSF is likely not required for the transcriptional activation of heat shock genes but probably is involved in the deactivation of HSF from its active state (48).

Additional studies with other inhibitors of ATP production should give us insight as to whether salicylate's effects on the induction of HSF binding activity, the induction heat shock puffs, and the inhibition of heat shock gene transcription and HSF hyperphosphorylation can be ascribed to decreased ATP levels alone.

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Note Added in Proof—Cotto *et al.* (Cotto, J. J., Kline, M., and Marimoto, R. I. (1996) *J. Biol. Chem.* **271,** 3355–3358) have recently demonstrated that salicylate treatment of human HeLa cells induces HSF1 binding without HSF1 hyperphosphorylation.

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