cases, indistinguishable from the quasicrystal model and thus lose their distinctive predictive value (*7*).

Although the nature of long-range order is the fundamental issue addressed in this article, its resolution is only one of many challenges in the field. The quasicrystal model predicts the long-range atomic order in the icosahedral alloys, but the detailed locations of the atoms in the structure need to be determined (*9*). The ultimate challenge, however, and the focus of present research in the field is to determine the structural, thermal, and electronic properties that characterize the new phase of matter (*8*).

REFERENCES AND NOTES

1. D. Shechtman, I. Blech, D. Gratias, J. W. Cahn, *Phys. Rev. Lett.* **53**, 1951 (1984).
2. D. Levine and P. J. Steinhardt, *ibid.*, p. 2477.
3. For a popular review of quasicrystals, see P. J. Steinhardt, *Am. Sci.* **74**, 586 (1986).
4. D. Shechtman and I. Blech, *Metall. Trans.* **16A**, 1005 (1985).
5. P. W. Stephens and A. I. Goldman, *Phys. Rev. Lett.* **56**, 1168 (1986); *ibid.* **57**, 2331 (1986).
6. L. Pauling, *Nature (London)* **317**, 512 (1985); *Phys. Rev. Lett.* **58**, 365 (1987).
7. The term "icosahedral glass model" has come to have different meanings to different scientists. Some scientists use the term "icosahedral glass model" to refer to icosahedrally symmetric solids with quenched positional disorder, independent of whether it comes from a well-defined microscopic packing rule. This allows for a continuum of possible structures, including a quasicrystal with quenched phason strains. With such a broad definition, the distinction between icosahedral glasses and quasicrystals is totally semantic. For this article, though, the term is used in the sense intended by P. W. Stephens and A. I. Goldman (*5*), who coined the expression, and by researchers who have constructed candidate models; that is, to refer to models in which positional disorder is introduced as a result of a fundamental interaction between microscopic clusters that results in a random packing rule. (I include the possibility of packing rules that differ from those already published in the literature.) In this case, there is a legitimate scientific distinction between the two models. The disorder produced by random packing rules takes a specific form that has a well-defined experimental signature and that is generally quite different from the predictions for quasicrystals, as is outlined in this article.
8. For an introduction and review, see P. J. Steinhardt and S. Ostlund, *The Physics of Quasicrystals* (World Scientific, Singapore, 1987), chap. 4.
9. C. L. Henley, *Comments Condensed Matter Phys.* **13**, 59 (1987).
10. R. Penrose, *Inst. Math. Appl. Bull.* **10**, 266 (1974).
11. V. Elser, in *Proceedings of the XVth International Colloquium on Group Theoretical Methods in Physics*, R. Gilmore and D. H. Feng, Eds. (World Scientific, Singapore, 1987).
12. See Scientific Correspondence, *Nature (London)* **319**, 102 (1986).
13. Issue on the International Workshop on Aperiodic Crystals, D. Gratias and L. Michel, Eds., *J. Phys. (Paris)* **47** (C3) (1986).
14. P. A. Bancel, P. A. Heiney, P. W. Stephens, A. I. Goldman, P. M. Horn, *Phys. Rev. Lett.* **54**, 2422 (1985).
15. P. A. Bancel and P. A. Heiney, *ibid.*, in press; J. D. Budai *et al.*, *ibid.* **58**, 2304 (1987).
16. T. C. Lubensky, J. E. S. Socolar, P. J. Steinhardt, P. A. Bancel, P. A. Heiney, *ibid.* **57**, 1440 (1986).
17. For a review and references on elasticity theory for quasicrystals, see T. C. Lubensky, in *Aperiodic Crystals*, M. V. Jaric, Ed. (Academic Press, Boston, in press).
18. W. Malzfeldt *et al.*, *J. Phys. (Paris)* **47** (C3), 379 (1986).
19. J. E. S. Socolar and D. C. Wright, *Phys. Rev. Lett.* **59**, 221 (1987).
20. P. J. Steinhardt, *ibid.* **57**, 2769 (1986); P. W. Stephens and A. I. Goldman, *ibid.*, p. 2270.
21. B. Dubost, J.-M. Lang, M. Tanaka, P. Sainfort, M. Audier, *Nature (London)* **324**, 48 (1986).
22. E. R. Ryba, *Earth Miner. Sci.* **55**, 51 (1986).
23. W. Ohashi and F. Spaepen, unpublished results.
24. A. R. Kortan, H. S. Chen, J. Waszczak, *J. Mater. Res.* **2**, 294 (1987).
25. P. M. Horn *et al.*, unpublished results.
26. S. J. Poon *et al.*, unpublished results.
27. Y. Shen *et al.*, *Phys. Rev. Lett.* **58**, 1440 (1987).
28. A. Garg and D. Levine, unpublished results.

29. I thank P. A. Heiney, J. E. S. Socolar, T. C. Lubensky, and D. C. Wright for discussions and for reviewing the manuscript, P. A. Bancel for providing Fig. 1, P. Horn for providing Fig. 4, and F. Spaepen for providing Fig. 5. This work has been supported in part by the National Science Foundation, Materials Research Laboratory Program, under grant DMR-8519059.

Research Articles

Purification and Properties of *Drosophila* Heat Shock Activator Protein

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Drosophila **heat shock activator protein, a rare trans-acting factor which is induced upon heat shock to bind specifically to the heat shock regulatory sequence in vivo, has been purified from shocked cells to more than 95 percent homogeneity by sequence-specific duplex oligonucleotide affinity chromatography. The purified protein has a relative molecular mass of 110 kilodaltons, binds to the regulatory sequence with great affinity and specificity, and strongly stimulates transcription of the *Drosophila hsp70* gene. Studies with this regulatory protein should lead to an understanding of the biochemical pathway underlying the heat shock phenomenon.**

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THE HEAT SHOCK RESPONSE WAS ORIGINALLY DISCOVERED in *Drosophila* (*1*) as the coordinate activation of a small number of cytogenetic loci in response to heat or chemical shock; it is now extensively studied as an evolutionarily conserved response to stress in all living species (*2*). The molecular analysis of heat shock gene activation has been advanced by genetic and biochemical approaches. Deletion mapping studies of the transcriptional regulation of these genes have identified a cis-acting heat shock control element (HSE), whose consensus sequence is CT-GAA--TTC-AG (*3*); sequences matching this heat-shock consensus sequence (HSC) are found in one or several copies upstream of the transcriptional start site.

Studies in our laboratory of protein-DNA interactions in vivo have identified a trans-acting factor referred to as heat shock activator protein (*4*), which is induced to bind specifically to the HSE only upon heat shock stimulation. HSE's of the noninduced genes are free of the protein and lie within nuclease hypersensitive sites in chromatin near the 5′ termini of these genes (*4, 5*). Heat shock activator protein can be extracted from nuclei of heat shock–

induced cells, and the extracted protein can bind in vitro to the vacant HSE of nuclear chromatin (*6*) as well as to the HSE sequence on a cloned DNA fragment (*7*). Binding competition studies indicate that the same factor is able to bind to HSE's of all heat shock genes. Activator protein preexists in normal cells without its HSE-binding ability, which is reversibly induced upon heat or chemical shock in the absence of de novo protein synthesis (*8*). This reversible change is likely to occur through a post-translational modification, the nature of which defines a new regulatory switch in the path to heat shock gene activation. A factor that also binds specifically to the HSE has been studied by Parker and co-workers by in vitro DNA-binding and transcription assays (*9–11*).

In order to purify heat shock activator protein, we used duplex oligonucleotide affinity chromatography, a general technique for the purification of sequence-specific DNA-binding proteins. The purified protein has a relative molecular mass of 110 kD (kilodaltons), as measured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The identification of the 110-kD protein was confirmed by a photo-affinity labeling procedure that can also be extended to crude nuclear extracts by combination with the electrophoretic mobility shift technique. The purified 110-kD heat shock activator stimulates transcription of a *hsp70* gene when microinjected in *Xenopus* oocytes, and it binds with extremely high affinity ($K_D = 4 \times 10^{-12} M$) to the heat shock consensus sequence. The protein also binds to *Escherichia coli* DNA, but with a six order of magnitude lower affinity.

Duplex oligo-affinity chromatography. The purification of DNA-binding proteins by chromatography has been facilitated by the development of sequence-specific DNA ligands (*12–15*). We designed a monomeric ligand that consists of a 30-bp binding site (the 14-bp HSE of a *hsp70* gene changed to fit the HSC perfectly, plus flanking DNA for binding stability) linked to a single-stranded spacer 12 nucleotides, or shorter, in length. The spacer also provides free amino groups for coupling to cyanogen bromide–activated Sepharose. Hence both ligand and spacer are prepared by simply annealing two synthetic oligonucleotides, one longer than the other. The concentration of binding sites per packed resin volume that is coupled to Sepharose is typically $2 \times 10^{-6} M$, several orders of magnitude above the dissociation constant (K_D) of known specific DNA-binding proteins.

Heat shock activator protein was purified by chromatography (Fig. 1A) on (i) heparin-Sepharose, (ii) HSC oligo-Sepharose, and (iii) FPLC (fast protein liquid chromatography) Mono S. Nuclei from 30 liters of *Drosophila* tissue culture cells (Schneider line 2, SL2) were isolated and then extracted with 0.35*M* NaCl (*16*). Specific binding was determined by exonuclease III (Exo III) protection with a 209-bp *hsp*82 promoter fragment end-labeled on the coding strand. Specific binding results in Exo III resistant DNA fragments of 130 and 127 nucleotides, which map to positions –91 (arrowhead on Fig. 1B) and –82 upstream of the transcriptional start, whereas digestion of free DNA with Exo III generates fragments of about one-half (or less) the original size (*7*).

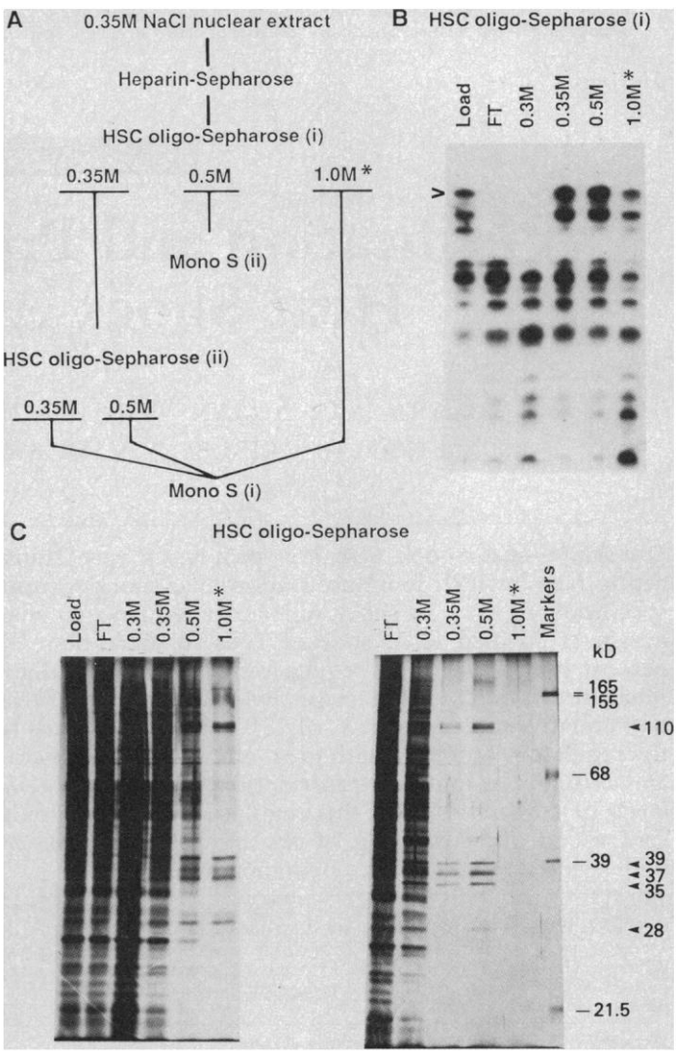

Fig. 1. (**A**) Purification scheme for heat shock activator protein. Schneider line 2 cells (SL2) were heat shocked at 37°C for 20 minutes and stored at −70°C. Nuclear extracts were prepared typically from 4×10^{10} cells by homogenization in two cell pellet volumes of solution I [10 m*M* Hepes, *p*H 7.9, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1 m*M* EGTA, 0.5 m*M* dithiothreitol (DTT), and 0.5 m*M* PMSF]. Nuclei were centrifuged at 1000*g* for 5 minutes, washed with two volumes of solution I, centrifuged again, resuspended in two volumes of solution II (10 m*M* Hepes *p*H 7.9, 0.4*M* NaCl, 1.5 m*M* $MgCl_2$, 0.1 m*M* EGTA, 0.5 m*M* DTT, 0.5 m*M* PMSF, 5 percent glycerol) and adjusted to 0.35*M* NaCl. The nuclei were extracted at 4°C for 30 minutes and centrifuged at 100,000*g* for 1 hour. The supernatant was dialyzed in solution III (20 m*M* Hepes, *p*H 7.9, 50 m*M* NaCl, 0.1 m*M* EDTA, 0.5 m*M* DTT, 0.5 m*M* PMSF, 20 percent glycerol), clarified by centrifugation, frozen in liquid nitrogen and stored at −70°C. Nuclear extracts (equivalent to 1.5×10^{11} cells) were applied to a 200-ml heparin-Sepharose CL-6B column (6 × 10 cm) equilibrated in 0.2*M* NaCl chromatography buffer (CB) (20 mm Hepes, *p*H 7.9, 0.1 m*M* EDTA, 0.5 m*M* DTT, 10 percent glycerol, 0.5 m*M* PMSF). Protein was eluted with a 600-ml linear gradient of CB-NaCl (0.2 to 1.5*M*), and 10-ml fractions were assayed by Exo III (*7*). For the preparation of the HSC oligo-Sepharose, 30 and 21 optical density (OD) units, respectively, of the upper 5′ GTCGACG-GATCCGAGCGCGCCTCGAATGTTCTAGAAAAGG 3′ and lower (the complementary sequence less 12 bases from the 3′ end) strands were annealed without prior gel purification in 400-μl of buffer containing 0.1*M* NaCl, 10 m*M* potassium phosphate, *p*H 8.2 by heating to 85°C for 2 minutes, transferring to a 70°C bath and then cooled to room temperature over several hours. The complete 400-μl volume of DNA was added to about 4 ml of CNBr-Sepharose 4B (Pharmacia) in 10 m*M* potassium phosphate, *p*H 8.2, in a total volume of ~7 ml of the phosphate buffer. The coupling reaction and termination were done by the usual procedures (*29*). A complete protocol is available upon request (*30*). (**B**) Affinity chromatography. The active heparin-Sepharose fractions were combined, dialyzed against solution III until NaCl was 0.2*M*, and applied to a 4-ml HSC oligo-Sepharose column equilibrated in 0.2*M* NaCl CB. Protein was eluted with 4 ml each of CB–0.3, 0.35, 0.5, and 1.0*M** NaCl, and HSE-binding activity was assayed. (**C**) SDS-PAGE and silver staining (*31*) of fractions from the oligo-Sepharose column. The 0.35*M* NaCl fraction of panel (i) was diluted to 0.2*M* NaCl with CB and applied to a 0.6-ml oligo-Sepharose column. The column was washed with four-column volumes of 0.3*M* NaCl-CB, eluted with 0.75-ml steps of CB–0.3, 0.35, 0.5, and 1.0*M* NaCl, and fractions were visualized by SDS-PAGE and silver staining (panel ii).

Nuclear extract was applied to a heparin-Sepharose column and eluted with a linear NaCl gradient. The HSE-binding activity was primarily in the heparin-Sepharose column fractions 35 to 43 (0.48 to 0.68*M* NaCl) (*15*). These fractions were pooled, applied on a 4-ml HSC oligo-Sepharose column in a moderate salt concentration of 0.2*M* NaCl to retard nonspecific binding, and heat shock activator was eluted with increasing concentrations of NaCl. The specific binding was eluted (Fig. 1B) at 0.35*M*, 0.5*M*, and 1.0*M** NaCl; in this initial elution an insufficient amount (one column volume) of each NaCl concentration was used, and as a result some activity trailed from the lower to the adjacent higher concentration. Hence, activity in the 1.0*M** fraction is trailed from the 0.5*M* fraction. With increasing NaCl, the protein complexity, as visualized by SDS-PAGE and silver staining (Fig. 1C, i), was greatly simplified. The 1.0*M** NaCl fraction included 110-, 39-, 37-, 35-, and 28-kD proteins. The 0.35*M* fraction was rechromatographed on HSC oligo-Sepharose, but 1.25 column volumes of NaCl were used in each step. The same 110- to 28-kD proteins were present in the step fractions 0.35*M* and 0.5*M* NaCl (Fig. 1C, ii) which contain binding activity (*15*). The 0.35*M* and 0.5*M* NaCl concentrations reproducibly elute the binding activity in subsequent purifications.

Fractions from HSC oligo-Sepharose column at (i) 1.0*M** and at (ii) 0.35*M*, and 0.5*M* NaCl were pooled and chromatographed on an FPLC Mono S ion-exchange column and eluted with a NaCl gradient. The activity across the column was assayed by nitrocellulose filter binding, Exo III protection, and gel shift techniques, and the protein profile was visualized by SDS-PAGE (Fig. 2A). The 110-kD protein and the HSE-binding activity peak together in Mono S fractions 22 and 23 (0.28*M* NaCl) with lesser activity and 110-kD protein in the fractions on either side (*15*). The 110-kD protein (actually a close doublet band reproducibly observed by SDS-PAGE) represents more than 95 percent of silver-stainable protein. The 39-, 37-, 35-, and 28-kD proteins as well as some smeared proteins of ~165-kD elute in fractions 36 to 41 (0.4*M* to 1.0*M* NaCl) and are clearly separated from the 110-kD protein and the peak of HSE-binding activity (*15*). Similar results are obtained when the HSC oligo-Sepharose (i) fraction (0.5*M* NaCl) was chromatographed on the Mono S column. The upper and lower bands of the 110-kD doublet have so far not been separable from each other and from the HSE-binding activity by further chromatography; we refer to both bands as the 110-kD protein.

We confirmed the identity of the 110-kD protein by quantitatively titrating Mono S fraction 23 with a single-site HSC of known concentration in a filter-binding assay (Fig. 2B). In this assay the binding is highly specific for the HSC; no interaction is observed with an unrelated duplex oligonucleotide of similar length (*15*). The concentration of active heat shock activator in fraction 23, $0.9 \times 10^{-8}M$, is compatible with that of the 110-kD protein, $2 \times 10^{-8}M$, but not with the protein concentration of the several smaller proteins, which represent less than 5 percent of protein in Mono S fractions 22 and 23. In more than ten independent purifications, this 110-kD protein is the only one at the end of the procedure. Purified heat shock activator protein is very stable; 50 to 100 percent of HSE-binding activity in Mono S fraction 22 is retained after 16 months of storage at −70°C. The yield of activity is good (roughly 50 percent) at each purification stage, except in the Mono S column (~80 percent loss), which results in a cumulative yield of 7 percent (Table 1). This low cumulative yield has now been

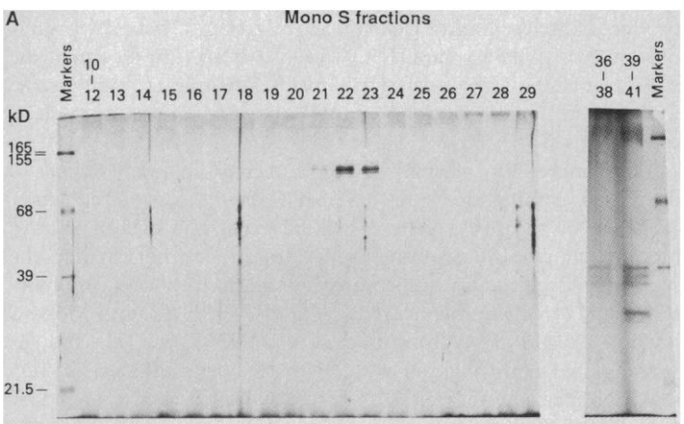

Fig. 2. Mono S chromatography. The 0.35 and 0.5*M* NaCl fractions from the second affinity column and the 1.0*M* NaCl fraction from the first column were combined, dialyzed in 0.1*M* NaCl–solution III, and applied to a 1-ml FPLC Mono S column (Pharmacia). Protein was eluted by a CB-NaCl gradient from 0.1 to 0.4*M*. The 0.5*M* fraction from the first column was purified similarly. (**A**) SDS-PAGE analysis. A portion (15 μl) of each fraction was added to a 4-μl mixture of 5 percent SDS, 0.3*M* tris-HCl, *p*H 6.8, and 50 m*M* DTT, and boiled for 2 minutes before loading on a 10 percent polyacrylamide gel (4 percent stacking gel) (*32*). After electrophoresis, the gel was stained with silver (*31*), and the protein content was estimated by comparison with markers (kilodaltons) of known concentration. (**B**) Titration of binding activity of purified heat shock activator protein. The synthetic duplex HSC sequence of Fig. 1C was gel-purified and labeled to 5×10^7 cpm/μg by filling the single-stranded region. Increasing amounts of Mono S fraction 23 was added to [^{32}P]HSC (4.5×10^4 cpm; 0.93 ng) in a 100-μl volume (final [^{32}P]HSC concentration was 0.36 n*M*) of filter binding buffer (FBB; 0.1*M* NaCl, 3 m*M* $MgCl_2$, 0.5 m*M* DTT, 15 m*M* tris-HCl, *p*H 7.4, 5 percent glycerol) containing 50 ng of fragmented *E. coli* DNA, and 10 μg of bovine serum albumin (BSA). After incubation for 20 minutes at 25°C, FBB (200 μl) was added, mixed, and 100-μl portions were spotted on each of three wet nitrocellulose filters, and filtered at reduced pressure. The filters were washed twice with 150 μl of FBB, dried, and counted. The bound radioactivity (280 cpm) of a blank filtration was subtracted from the experimental readings, which were averaged over the triplicate filtrations. The binding curve shown was at a plateau at ~3,400 cpm, and since the total input was 15,000 cpm per filter, the retention efficiency of nitrocellulose for activator protein under our conditions was 23 percent. At half-maximal binding, the concentration of activator molecules is 0.18 n*M*, half the input DNA concentration, from which the concentration of activator in Mono S fraction 23 is calculated to be $0.9 \times 10^{-8}M$.

Table 1. Purification efficiency of heat shock activator protein. One unit of activity is defined as the amount of activator that binds 1 ng of the *hsp82* promoter in an Exo III protection assay (*7*) carried out under conditions of DNA excess ($5 \times 10^{-10}M$). ND, not determined.

Fraction	Total protein (mg)	Specific activity (U/mg)	Total units	Recovery (%)	Purification factor
0.35*M* nuclear extract (1.5×10^{11} cells/30 l)	401.5	13.6	5,476	100	1
Heparin-Sepharose	55.3	68.2	3,770	68.8	5
HSC oligo-Sepharose (i)					
0.35*M*	0.6	420	252	4.6	31
0.5*M*	0.1	8,990	899	16.4	661
1.0*M**	0.036	19,083	687	12.5	1,403
HSC oligo-Sepharose (ii)					
0.35*M* + 0.5*M*	0.036	ND	ND	ND	ND
Mono S (i) + (ii)	0.004	95,750	383	7.0	7,040

*As corrected for step fraction volume.

improved, to ~20 percent, by the use of very small Mono S column volumes. The actual number of 110-kD monomers in the cell by a simple calculation [(amount of purified activator × Avogadro's number)/molecular size × initial cell number × yield) = (4×10^{-6} g × 6×10^{23} molecules per mole)/(1.1×10^5 g/mol × 1.5×10^{11} cells × 0.07)] is about 2000 active 110-kD monomers per cell, if we assume complete extraction from nuclei with 0.35*M* NaCl and no leakage to the cytosol. We observed that 0.35*M* NaCl results in nearly complete extraction from nuclei and that less than 50 percent of the total HSE-binding activity can be found in the cytosol (*17*). Hence 2000 to 4000 110-kD monomers per cell is a reasonable estimate of the total cellular concentration after 15 minutes of heat shock stimulation at 37°C, a point at which the binding activity is at a maximum level (*8*). Starting from the 0.35*M* NaCl nuclear extract, the overall increase in specific activity (purification factor) is calculated to be ~7000-fold, a value comparable to the purification factor for the transcription factor Spl [~6000-fold, calculated in the same way from the HeLa nuclear extract (*14*)], but not to that recently reported for the 70-kD heat shock transcription factor (*11*) (250,000-fold purification with 83 percent yield) (*18*).

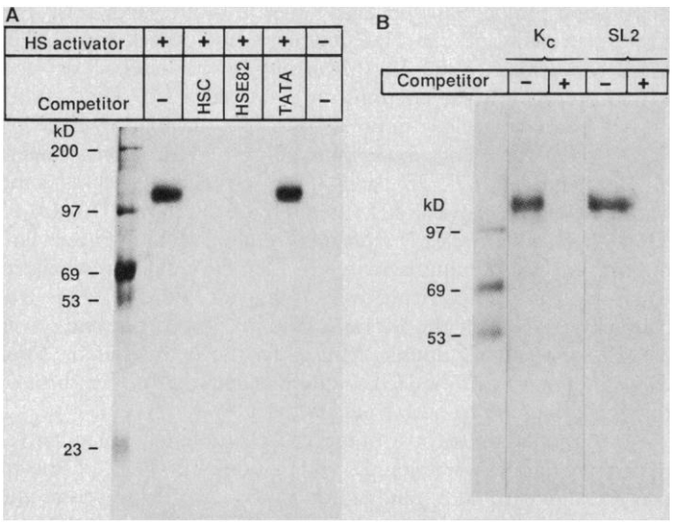

Fig. 3. (**A**) Photo-affinity labeling of purified heat shock activator protein. A 50-bp sequence containing the HSE 82 was labeled by annealing the upper strand (see Fig. 6A, positions −94 to −45) with a 15-base complementary primer (−59 to −45) and filling in the lower strand with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP, dATP, dGTP, and a 1:1 ratio of dTTP:BrdU following standard procedures. ^{32}P-labeled BrdU–substituted HSE 82 (0.2 ng) was incubated with 100 ng of *E. coli* DNA, 2.5 μg of β-lactoglobulin, and 8 ng (an excess) of Mono S fraction 22 in a 13-μl volume for 10 minutes at 25°C. The open microfuge tubes were placed under (~5-cm sample to lamp separation) an inverted 302-nm ultraviolet transilluminator (UV Products Model TM 40) and irradiated at 25°C for 20 minutes. A solution of 0.5 μl of 0.1*M* $CaCl_2$ and 1.0 μl of a mixture of DNase I and micrococcal nuclease (2.5 U/μl and 0.2 U/μl, respectively, in 5 m*M* tris-HCl, *p*H 7.5, 50 m*M* NaCl, 35 percent glycerol, BSA at 50 ng/μl, 10 m*M* $CaCl_2$) was added and digested for 10 minutes at 30°C. The reaction was terminated with 0.8 μl of 0.5*M* Na_2EDTA. We added 4 μl of 5 percent SDS, 0.3*M* tris-HCl, *p*H 6.8, 20 percent glycerol, and dye; the sample was boiled for 2 minutes and subjected to SDS-PAGE and autoradiography. The dye front of the gel, containing undigested oligonucleotides was excised. The competitor DNA was (i) HSC, 25 ng of the HSC sequence shown in Fig. 1C without the single-stranded region; (ii) HSE 82, 25 ng of the *hsp82* upstream sequence of Fig. 6A (44 bp, positions −94 to −51); and (iii) TATA, 80 ng of *hsp70* gene sequence from −46 to −2 (upper strand) and −46 to −10 (lower strand) (*33*). (**B**) Photo-affinity labeling of heat-shock activator in crude nuclear extracts. Nuclear extracts were prepared from heat-shocked SL2 cells and K_c cells (*34*). Nuclear extract (30 μl) was mixed with 1.5 ng of [^{32}P]BrdU-HSE 82, 150 μg of BSA, 2 μg of *E. coli* DNA, and 15 μg of poly(dI-dC) · poly(dI-dC), with or without 37.5 ng of unlabeled HSC competitor, and NaCl was added to 140 m*M*; the final volume was adjusted to 40 μl and incubated for 10 minutes at 25°C. The mixture was subjected to electrophoresis on a 0.5-cm thick horizontal 1 percent low-melting agarose gel (Seaplaque, Marine Colloids) in 45 m*M* tris-borate EDTA at 4°C for ~1.5 hours until the bromphenol blue front migrated about 5 cm. The gel was placed over Saran Wrap on the filter surface of the 302-nm ultraviolet transilluminator at 4°C and irradiated for 7 minutes, and autoradiographed for 30 to 60 minutes. The region corresponding to the specific protein-DNA complex, or its equivalent in the samples with competitor DNA, was located by inspection (the bands are more diffuse than the examples in Fig. 6A) and excised. To each slice 10 μl of a mixture of 0.3*M* tris-HCl, *p*H 6.8, 6 percent SDS, 15 percent glycerol, 70 m*M* DTT, was added, and the samples were boiled for 2 minutes. The warm liquid samples were loaded on a 1.5 mm thick, 8 percent polyacrylamide gel (4 percent stacker) before application of electrophoresis buffer.

Photo-affinity labeling. We have confirmed the 110-kD value for heat shock activator protein by a rigorous and independent method. Lin and Riggs (*19*) showed that lac repressor can be specifically cross-linked by ultraviolet irradiation to BrdU (5′-bromo-2′-deoxyuridine triphosphate)-substituted lac operator DNA. The ultraviolet light displaces bromine from the BrdU and produces a free radical; a vicinal reactive group of bound repressor protein cross-links to the DNA. Chodosh *et al.* (*20*) measured the molecular size of the adenovirus major late transcription factor; DNA substituted in vitro and a partially purified protein from HeLa cells was used.

To promote more efficient binding and cross-linking, we chose to do a BrdU substitution and ^{32}P-labeling in the natural regulatory sequence of the *hsp82* gene (HSE 82) that has highest relative binding affinity. Substituting with BrdU does not change the specific binding activity as measured by the electrophoretic mobility shift assay (*15*). We then mixed [^{32}P]BrdU-HSE 82 with Mono S fraction 22 and cross-linked under ultraviolet light. The complex was digested with nuclease with the subsequent addition of SDS sample buffer; the digestion mixture was boiled and separated by SDS-PAGE. In Fig. 3A we show unequivocally that the 110-kD

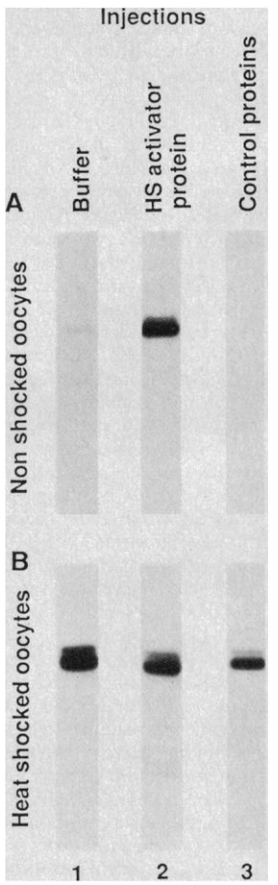

Fig. 4. Primer extension analysis of RNA synthesized in *Xenopus laevis* oocytes after injection of heat shock activator protein. Stage 6 *Xenopus* oocytes were isolated by treating with collagenase (*35*). A 10-nl sample of closed circular plasmid 122X14 DNA at 250 μg/ml in 88 m*M* NaCl were injected into the nucleus of each oocyte (*36*). Plasmid 122X14 contains a complete *hsp70* gene and flanking DNA from locus 87A (*33*). After incubation for 4 to 7 hours, groups of 40 oocytes were each injected a second time into the nucleus with either 30 nl of buffer (0.3*M* NaCl-CB), purified heat shock activator at 40 μg/ml, or a Mono S column fraction of equivalent protein concentration that lacked HSE-binding activity (control proteins). After a 2-hour incubation at 22°C, half of each group of oocytes was left at room temperature (**A**), while the other half was heat shocked at 34°C for 90 minutes (**B**). The total oocyte RNA was purified (*37*); transcripts of the injected *Drosophila hsp70* gene were analyzed by primer extension with a ^{32}P end-labeled oligonucleotide complementary to positions +149 to +177 of the mRNA (*33*). Primer (5 ng) was hybridized with 5 μg of oocyte RNA by heating to 70°C for 3 minutes and cooling to 37°C for 30 minutes. The primer was extended with AMV reverse transcriptase for 30 minutes at 37°C, and the complementary DNA was analyzed on a 5 percent sequencing gel. The autoradiograph in (A) was exposed three times longer than the one in (B).

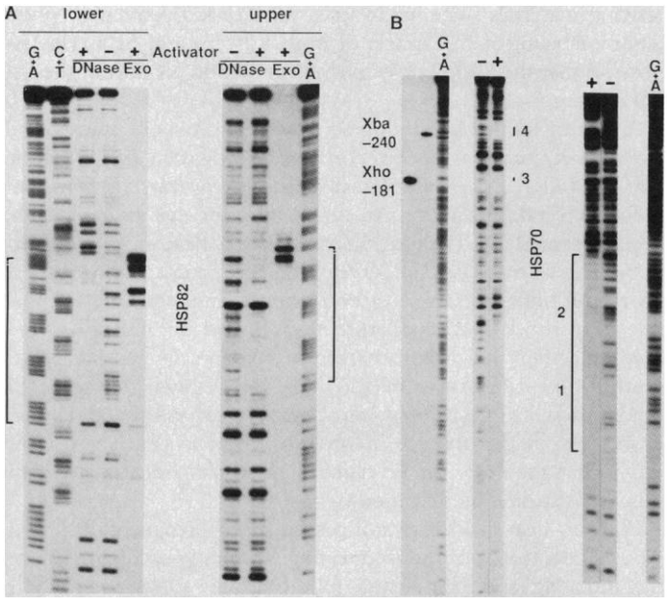

Fig. 5. (**A**) DNase I and Exo III protection assays of purified heat shock activator protein. An *hsp82* gene promoter fragment (*7*) (positions −170 to +39) was ^{32}P-labeled at the 5′ end of either the upper or lower strand. About 1 ng of [^{32}P]DNA was incubated for 15 minutes at 25°C with 200 ng of *E. coli* DNA, 2 μg of BSA, and 10 ng of Mono S fraction 22 in "binding buffer" (*7*) (except that NaCl was 100 m*M*) in a final volume of 50 μl. Exo III (300 U) or DNase I (1.3 U) was added, and the reaction was incubated for 6 minutes at 30°C, or 2 minutes at 24°C, respectively, and then terminated by the addition of SDS to 0.5 percent and Na_2EDTA to 10 m*M*. The DNA was purified, dissolved in 5 μl of formamide-dye mixture, and subjected to electrophoresis on a 6 percent sequencing gel. (**B**) An *hsp70* gene promoter (Sal I to Bgl I) fragment [from plasmid 122X14 (*33*)] was 5′ end-labeled with ^{32}P at the Sal I site. DNase I protection assays were performed as in (A), except that the volume of the initial binding reaction was 25 μl. An equal volume of 100 m*M* NaCl binding buffer (*7*) was then added, and DNase I digestion was initiated. Equal portions of purified DNA were subjected to electrophoresis on a 5 percent (left panel) and an 8 percent (right panel) sequencing gel to further resolve the footprints over the four separate HSE's of *hsp70*. The two distal HSE's (HSE 3 and 4) are located directly at Xho I and Xba I sites, respectively, as indicated by restriction fragments.

protein is labeled by ^{32}P transfer from the [^{32}P]BrdU-HSE 82 (the overall size is slightly increased to ~116 kD by the covalently attached oligonucleotides remaining after nuclease digestion). The photo-affinity labeling is specific for HSE sequences only. In a competition experiment, unlabeled HSC or HSE 82 abolishes the signal, whereas a different duplex oligonucleotide containing the *hsp70* gene TATA box does not. Withholding of purified activator protein from the reaction also fails to produce any signal.

We have also demonstrated specific photo-affinity labeling of heat shock activator protein in crude nuclear extracts by electrophoretic separation of the specific protein-DNA complex from unbound or nonspecifically bound [^{32}P]BrdU-HSE 82 on a low-melting-agarose gel, cross-linking in situ, and analysis of the specific complex by SDS-PAGE. Specific labeling of a 110-kD protein in nuclear extracts prepared from two different lines (K_c, SL2) of heat-shocked *Drosophila* tissue culture cells is observed in Fig. 3B. As would be expected, a similar experiment with nonshocked cell extracts shows relatively little to no labeling of the 110-kD protein (*15*). We also found specific labeling of the 110-kD protein in the active fractions of the heparin-Sepharose and HSC oligo-Sepharose column (*15*). The fact that only the 110-kD protein is specifically cross-linked to [^{32}P]BrdU-HSE 82 at all stages of purification supports the identity of the 110-kD protein as intact heat shock activator and renders unlikely (but does not exclude) the possibility that the 110-kD protein might itself be processed from a larger precursor. We have applied the photo-affinity labeling technique to visualize the size of heat shock activator protein in crude extracts of yeast and human cells. The activator in these different species have large but dissimilar size (*21*). In an independent study, Khoury and colleagues [K. T. Jeang *et al.* (*22*)] have also effectively combined ultraviolet cross-linking of protein-DNA complexes with the electrophoretic mobility shift procedure on polyacrylamide gels.

Transcriptional activation. Transcriptional activation of a microinjected *Drosophila* heat shock gene was previously shown in *Xenopus* oocytes subjected to heat shock (*23*). Here we have assayed the functional properties of purified heat shock activator protein by microinjection into nonshocked *Xenopus* oocytes. Transcription of a microinjected *Drosophila hsp70* gene is indeed stimulated by subse-

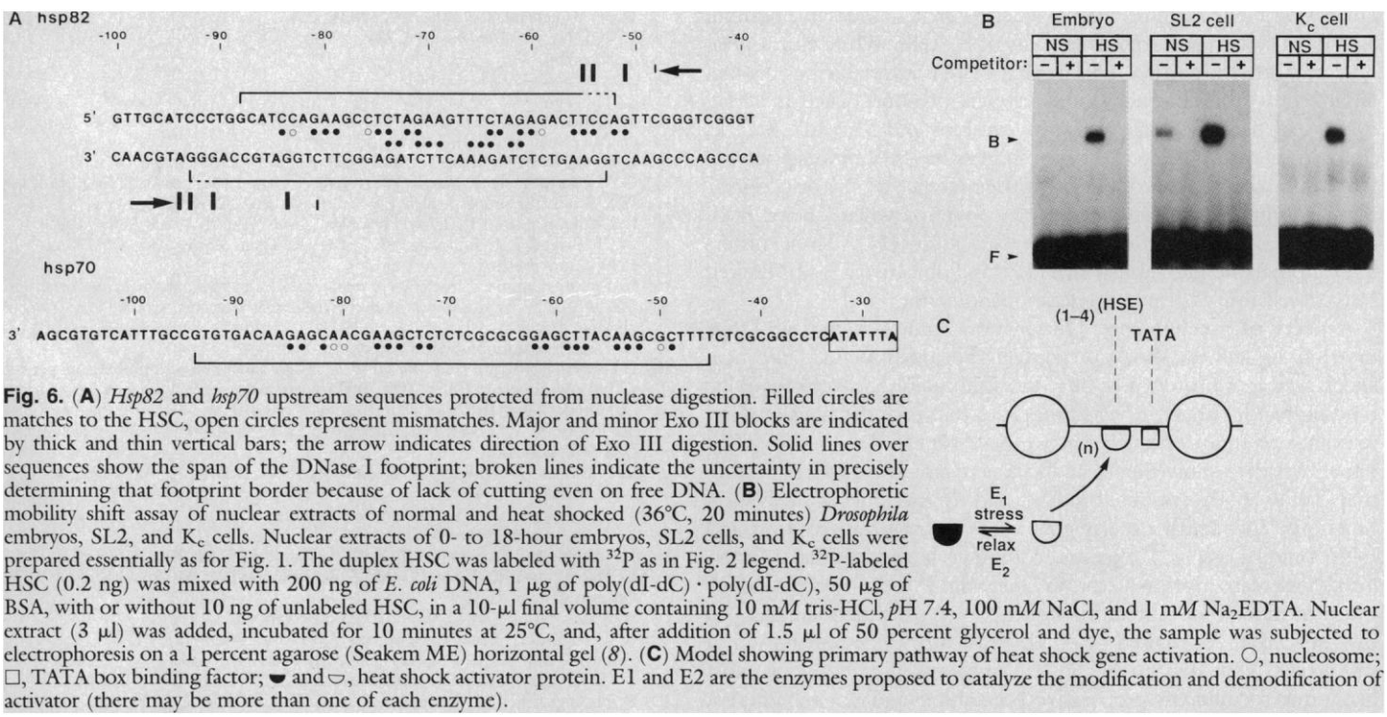

Fig. 6. (**A**) *Hsp82* and *hsp70* upstream sequences protected from nuclease digestion. Filled circles are matches to the HSC, open circles represent mismatches. Major and minor Exo III blocks are indicated by thick and thin vertical bars; the arrow indicates direction of Exo III digestion. Solid lines over sequences show the span of the DNase I footprint; broken lines indicate the uncertainty in precisely determining that footprint border because of lack of cutting even on free DNA. (**B**) Electrophoretic mobility shift assay of nuclear extracts of normal and heat shocked (36°C, 20 minutes) *Drosophila* embryos, SL2, and K_c cells. Nuclear extracts of 0- to 18-hour embryos, SL2 cells, and K_c cells were prepared essentially as for Fig. 1. The duplex HSC was labeled with ^{32}P as in Fig. 2 legend. ^{32}P-labeled HSC (0.2 ng) was mixed with 200 ng of *E. coli* DNA, 1 μg of poly(dI-dC) · poly(dI-dC), 50 μg of BSA, with or without 10 ng of unlabeled HSC, in a 10-μl final volume containing 10 m*M* tris-HCl, *p*H 7.4, 100 m*M* NaCl, and 1 m*M* Na_2EDTA. Nuclear extract (3 μl) was added, incubated for 10 minutes at 25°C, and, after addition of 1.5 μl of 50 percent glycerol and dye, the sample was subjected to electrophoresis on a 1 percent agarose (Seakem ME) horizontal gel (*8*). (**C**) Model showing primary pathway of heat shock gene activation. ○, nucleosome; □, TATA box binding factor; ◒ and ◡, heat shock activator protein. E1 and E2 are the enzymes proposed to catalyze the modification and demodification of activator (there may be more than one of each enzyme).

quent injection of purified heat shock activator into *Xenopus* oocytes (Fig. 4); the start of the *Drosophila hsp70* transcript is identical with *hsp70* transcripts made in vivo in shocked *Drosophila* cells (*24*). In contrast, a low level of transcription is observed in oocytes injected with buffer or control proteins from a Mono S column fraction lacking HSE-binding activity. The transcriptional stimulation by purified heat shock activator as measured by the primer extension assay is about 30-fold, when compared to *hsp70* transcripts made in oocytes injected with buffer. Heat shock activator protein does not stimulate the transcription of a *Herpes simplex* thymidine kinase gene microinjected in oocytes (*24*). Finally, we show that the microinjected *hsp70* gene in oocytes injected with buffer or control proteins can be transcriptionally activated, since an external heat shock stimulus does result in high levels of *hsp70* transcription.

Binding affinity and specificity. We measured quantitatively the affinity and specificity of purified heat shock activator protein for the (single) HSC by following closely the nitrocellulose filter-binding procedures and calculations developed originally for lac repressor (*25*). Assuming that one heat shock activator molecule binds to one single site HSC, we calculate the dissociation constant, K_D, for HSC to be $4 \times 10^{-12} M$ in 0.1*M* NaCl, 3 m*M* $MgCl_2$ and the K_D for *Escherichia coli* DNA to be $2 \times 10^{-6} M$. Hence the specificity K_D(DNA)/K_D(HSC) of activator for the HSC over other (*E. coli*) sequences is $\sim 10^6$. The binding is stable; the half-life of the complex is 30 to 40 minutes at 25°C (*15*).

The specific binding of purified activator protein to the natural promoters of the *hsp82* and *hsp70* genes is shown by deoxyribonuclease I (DNase I) and Exo III protection (footprinting) experiments (Fig. 5). Using either nuclease as structural probe, we find that the protected region in the *hsp82* promoter lies over the three overlapping HSE's (HSE 82) characteristic to this gene (Figs. 5A and 6A), consistent with previous mapping results in vivo (*4*) and in vitro (*7*) with unpurified extracts. The purified activator protein also generates specific footprints over the four separate HSE's in the region upstream of the *hsp70* gene (Fig. 5B).

The inducible binding of heat shock activator protein in vivo has been previously shown (*4*) as has the higher in vitro binding activity of extracts of heat shocked SL2 cells. However, a constitutive binding activity has been described by others in K_c cells, another *Drosophila* cell line (*9*). In Fig. 6B we show that an inducible binding activity is clearly observed in extracts of K_c cells. While electrophoretic mobility shift assays indicate that there is a variable but distinct basal level of binding activity in extracts of nonshocked material, extracts of heat shocked *Drosophila* embryos and SL2 and K_c cells show significantly (10 to 20 times) higher levels of binding activity that can be specifically competed by the presence of unlabeled HSC. Similar inducible binding behavior is observed when labeled HSE 82 DNA is used in the mobility shift assay (*15*). These results indicate that the inducibility of HSE-binding activity is not limited to SL2 cells but is a more general phenomenon.

Aspects of mechanisms. Our in vivo studies of protein-DNA interactions at heat shock promoters previously defined the heat shock activator protein as a heat shock inducible, sequence-specific binding factor. These in vivo properties reinforce the physiological relevance of the properties that we have observed to be similar in vitro. Our data show that heat shock activator is a 110-kD protein that fulfills both specific binding and transcriptional activation functions. In parallel experiments with in vitro transcription and DNA-binding assays, Parker and colleagues have identified a factor, heat shock transcription factor, in *Drosophila* K_c cells, which binds to the same heat shock regulatory sequence, yet is distinctly smaller [43 kD (quoted in *26*) revised to 70 kD (*11*) during the preparation of this article]. The discrepancy in size of the purified factors is most likely due to different preparative procedures and not to different starting materials since we observe the 110-kD protein by photoaffinity labeling with extracts of both K_c cells and SL2 cells. It is possible that the 70-kD protein is a degradation product of the 110-kD protein.

A lack of inducible binding activity for heat shock transcription factor in K_c cells has been reported (*9*). However, Fig. 6B shows that K_c cells, as well as SL2 cells and *Drosophila* embryos, have inducible binding activity. An explanation for this discrepancy has been presented (*8*). The inducible property of heat shock activator is not restricted to *Drosophila*. *Xenopus*, chicken, human, and yeast cells grown on nonfermentable carbon sources similarly have an inducible heat shock activator protein (*21*), and an inducible HSE-binding activity has been observed in human cells without protein synthesis by Kingston *et al.* (*27*). We have shown that the HSE-binding activity of the *Drosophila* activator is reversibly and repeatedly induced in the absence of protein synthesis (*8*). These results imply that the basis for reversible inducibility lies in a reversible post-translational modification.

We view heat shock activator protein as final recipient of an initial stress signal that is transduced to the enzymes proposed to catalyze the inducing and deinducing modifications. Once induced, the direct binding of activator to the heat shock promoter poised for transcription by the presence of constitutively bound TATA factor (*4, 7*) and RNA polymerase II (*28*) could be sufficient for activation of the transcriptional apparatus. However, it is possible that a further heat shock modification separate from the binding function may also be required for transcriptional activation. In the model shown in Fig. 6B, we summarize our current understanding of the primary pathway to activation of heat shock genes. Further elaboration and elucidation of the model should be feasible with antibodies to heat shock activator protein, and with the cloning and expression of its gene.

REFERENCES AND NOTES

1. F. M. Ritossa, *Experientia (Basel)* **18**, 571 (1962); F. M. Ritossa, *Exp. Cell Res.* **35**, 601 (1964).
2. M. Ashburner and J. J. Bonner, *Cell* **17**, 241 (1979); M. J. Schlesinger, M. Ashburner, A. Tissieres, *Heat Shock* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982); S. Lindquist, *Annu. Rev. Biochem.* **55**, 1151 (1986); E. Craig, *CRC Critical Rev. Biochem.* **18**, 239 (1985); L. Nover, D. Hellmund, D. Neumann, K.-D. Scharf, E. Serfling, *Biol. Zentr.* **103**, 357 (1984).
3. H. R. B. Pelham, *Cell* **30**, 517 (1982).
4. C. Wu, *Nature (London)* **309**, 229 (1984).
5. ———, *ibid.* **286**, 854 (1980).
6. ———, *ibid.* **311**, 81 (1984).
7. ———, *ibid.* **317**, 84 (1985).
8. V. Zimarino and C. Wu, *ibid.* **327**, 727 (1987).
9. C. Parker and J. Topol, *Cell* **37**, 273 (1984).
10. J. Topol, D. M. Ruden, C. Parker, *ibid.* **42**, 527 (1985).
11. G. Wiederrecht, D. J. Shuey, W. A. Kibbe, C. S. Parker, *ibid.* **48**, 507 (1987).
12. P. J. Rosenfeld and T. J. Kelly, *J. Biol. Chem.* **261**, 1398 (1986).
13. J. T. Kadonaga and R. Tjian, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5889 (1986).
14. M. R. Briggs, J. T. Kadonaga, S. P. Bell, R. Tjian, *Science* **234**, 47 (1986).
15. C. Wu, unpublished observations.
16. In a pilot experiment, nuclei were extracted with increasing NaCl concentrations; 0.35*M* NaCl was the lowest concentration that gave quantitative extraction of binding activity.
17. V. Zimarino, unpublished observations.
18. However, a calculation for the number of heat shock transcription factors per cell would indicate only 50 to 100 70-kD monomers per cell nucleus, an order of magnitude lower than our estimate and a number too low to ensure adequate occupancy of all HSE's (estimated to be roughly 100) present in the diploid *Drosophila* genome.
19. S.-Y. Lin and A. D. Riggs, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 947 (1974).
20. L. A. Chodosh, R. W. Carthew, P. A. Sharp, *Mol. Cell. Biol.* **6**, 4723 (1986).
21. V. Zimarino, C. Tsai, C. Wu, in preparation.
22. K. T. Jeang *et al.*, in preparation.
23. R. Voellmy and D. Rungger, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1776 (1982); H. R. B. Pelham and M. Bienz, *EMBO J.* **1**, 1473 (1982); M. Bienz and H. R. B. Pelham, *ibid.*, p. 1583.
24. B. Walker and I. Dawid, unpublished observations.
25. A. D. Riggs, H. Suzuki, S. Bourgeois, *J. Mol. Biol.* **48**, 67 (1970); S.-Y. Lin and A. D. Riggs, *ibid.* **72**, 671 (1972); *Cell* **4**, 107 (1975).
26. S. Munro and H. Pelham, *Nature (London)* **317**, 447 (1985).
27. R. E. Kingston, T. J. Scheutz, Z. Larin, *Mol. Cell. Biol.* **7**, 1530 (1987).

28. D. S. Gilmour and J. T. Lis, *ibid.* **6**, 3984 (1986).
29. D. J. Arndt-Jovin *et al.*, *Eur. J. Biochem.* **54**, 411 (1975); H. Bunemann, P. Westhoff, R. G. Herrmann, *Nucleic Acids Res.* **10**, 7163 (1982).
30. C. Wu, C. Tsai, S. Wilson, in *Genetic Engineering*, J. K. Setlow and A. Hollaender, Eds. (Plenum, New York, in press), vol. 10.
31. M. G. Harrington *et al.*, *J. Electrophoresis* **5**, 236 (1984).
32. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
33. P. J. Mason, I. Torok, I. Kiss, F. Karch, A. Udvardy, *J. Mol. Biol.* **156**, 21 (1982); M. Goldschmidt-Clermont, *Nucleic Acids Res.* **8**, 235 (1980).
34. The K_c cells were originally obtained from the laboratory of David Hogness.
35. R. H. Reeder, J. G. Roan, M. Dunaway, *Cell* **35**, 449 (1983).
36. D. D. Brown and J. B. Gurdon, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2064 (1977).
37. T. D. Sargent, M. Janovich, I. B. Dawid, *Dev. Biol.* **114**, 238 (1986).
38. We thank C. Klee for assistance with the binding constant measurements; P. Wagner, C. Klee, I. Grummt, W. Garrard, P. de la Pena, and M. Zasloff for helpful discussions; P. Mason and B. Sauer for plasmid 122X14 and K_c cells, respectively; M. Harrington and C. Merril for the silver staining protocol; M. Brownstein for preparing synthetic oligonucleotides; B. Miller for secretarial assistance; and M. Singer for a critical review of the manuscript.

6 June 1987; accepted 10 October 1987

Antarctic Stratospheric Chemistry of Chlorine Nitrate, Hydrogen Chloride, and Ice: Release of Active Chlorine

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The reaction rate between atmospheric hydrogen chloride (HCl) and chlorine nitrate ($ClONO_2$) is greatly enhanced in the presence of ice particles; HCl dissolves readily into ice, and the collisional reaction probability for $ClONO_2$ on the surface of ice with HCl in the mole fraction range from ~0.003 to 0.010 is in the range from ~0.05 to 0.1 for temperatures near 200 K. Chlorine (Cl_2) is released into the gas phase on a time scale of at most a few milliseconds, whereas nitric acid (HNO_3), the other product, remains in the condensed phase. This reaction could play an important role in explaining the observed depletion of ozone over Antarctica; it releases photolytically active chlorine from its most abundant reservoir species, and it promotes the formation of HNO_3 and thus removes nitrogen dioxide (NO_2) from the gas phase. Hence it establishes the necessary conditions for the efficient catalytic destruction of ozone by halogenated free radicals. In the absence of HCl, $ClONO_2$ also reacts irreversibly with ice with a collision efficiency of ~0.02 at 200 K; the product hypochlorous acid (HOCl) is released to the gas phase on a time scale of minutes.

THE DEPLETION OF OZONE IN THE ANTARCTIC STRATOSPHERE that has occurred in the spring over the past 5 or 6 years has received considerable attention (*1*). Proposed theories to explain the ozone decline include dynamical uplifting of the lower stratosphere (*2*) and chemistry involving catalytic cycles with halogen-containing free radicals as chain carriers; McElroy *et al.* suggested one that includes the reaction of chlorine monoxide (ClO) and bromine monoxide (BrO) (*3*); Solomon *et al.* (*4*) and Crutzen and Arnold (*5*) proposed another one that incorporates hypochlorous acid (HOCl) photolysis and formation by the ClO + HO_2 reaction. Another potential cycle is:

$$ClO + ClO \rightarrow Cl_2O_2 \quad (1)$$

$$Cl_2O_2 + M \rightarrow Cl_2 + O_2 + M \quad (2)$$

$$Cl_2 + h\nu \rightarrow 2Cl \quad (3)$$

$$2(Cl + O_3 \rightarrow ClO + O_2) \quad (4)$$

$$\text{Net reaction: } 2O_3 \rightarrow 3O_2 \quad (5)$$

where M is a third-body molecule and $h\nu$ is a photon.

This cycle is similar to the one we proposed earlier (*6*), except that in the second step photolysis of the ClO dimer is replaced by thermal decomposition. Room-temperature, low-pressure studies of the ClO self-reaction (*7*) have shown that the four-center channel with chlorine (Cl_2) and O_2 as products proceeds at about the same rate as the channel that yields atomic chlorine (Cl) and chlorine dioxide (ClOO), which is slightly endothermic. Because of the larger entropy barrier, the activation energy for the thermal decomposition of the dimer should be smaller for the four-center process than for the simple bond-fission processes, which generate either Cl and ClOO or two ClO radicals. Thus at 180 K the four-center channel may dominate.

These theories require significant amounts of active chlorine to be liberated from the reservoir species [hydrogen chloride (HCl) or chlorine nitrate ($ClONO_2$) or both]; furthermore, low levels of nitrogen dioxide (NO_2) are needed, or else the ClO chain carrier would be scavenged to produce $ClONO_2$. To achieve these conditions, the following heterogeneous reactions have been proposed to occur on the surfaces of polar stratospheric clouds (*4*) (PSCs), which are believed to consist mainly of ice and nitric acid (HNO_3) (*5, 8, 9*):

$$ClONO_2 + HCl \rightarrow Cl_2 + HNO_3 \quad (6)$$

$$ClONO_2 + H_2O \rightarrow HOCl + HNO_3 \quad (7)$$

We present the results of laboratory experiments that explored the interactions of gas-phase $ClONO_2$ and HCl with ice crystals. A low

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