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Drosophila nuclear proteins bind to regions of alternating C and T residues in gene promoters

D Gilmour

G Thomas

Sarah C.R. Elgin
Washington University in St. Louis, selgin@wustl.edu

Drosophila Nuclear Proteins Bind to Regions of Alternating C and T Residues in Gene Promoters

DAVID S. GILMOUR,* GRAHAM H. THOMAS,† SARAH C. R. ELGIN

Proteins from *Drosophila* nuclei that bind to regions of alternating C and T residues present in the promoters of the heat shock genes *hsp70* and *hsp26* and the histone genes *his3* and *his4* have been purified. These proteins bind to isolated linear DNA, and genomic footprinting analyses indicate that they are bound to DNA in nuclei. In supercoiled plasmids at low *p*H, some of these DNA sequences adopt triple-helical structures which, if they form in vivo, could significantly affect chromatin structure. The nuclear proteins described here, and not necessarily the deformed conformation of the DNA, may be responsible for maintaining a potentially inducible promoter structure before transcriptional activation.

MOST DNA IN EUKARYOTES IS ORganized in ordered arrays of nucleosomes (*1*). However, discontinuities in these arrays, detected as deoxyribonuclease I (DNase I)–hypersensitive sites, are present in the promoters of active or inducible genes; such discontinuities may be important in promoter function (*2*). Certain DNA sequences readily become deformed from their normal right-handed double-helical conformation under appropriate conditions, and it has been suggested that such deformable sequences may contribute to chromatin structure by preventing the formation of nucleosomes (*3, 4*).

A 20-bp region of alternating C and T residues is present upstream of the *Drosophila his3* gene. In supercoiled plasmids at *p*H 5.5, this region became deformed from the normal double-helical conformation of B-form DNA, as detected by sensitivity to the single-strand cutting enzyme S1 nuclease (Fig. 1A, lane 2). The region appeared to have adopted the triple-helical conformation of H-form DNA (*5*), as the cuts localized on the purine strand were on one side of the mirror repeat (Fig. 1C).

While monitoring protein-DNA interactions in intact nuclei, we found evidence of an association of protein with the CT sequence in the histone promoter region. To analyze these protein-DNA interactions, we performed footprinting analyses (*6*) on nuclei from *Drosophila* embryos (*7*). Isolated nuclei were treated with DNase I, and the positions of the cuts were determined by high-resolution indirect end-labeling analysis; bound protein would be expected to protect the DNA from nuclease digestion. A comparison of the digestion pattern of the region upstream of *his3* produced in nuclei (Fig. 1B, lanes 3 and 7) to that of purified genomic DNA (Fig. 1B, lanes 2 and 6) showed that the CT region was resistant to digestion in chromatin. A distinct footprint was evident on the pyrimidine strand (Fig. 1B, lane 7), whereas the boundary on the distal side of the purine strand is less distinct

Department of Biology, Washington University, St. Louis, MO 63130.

*Present address: Department of Molecular and Cell Biology, Pennsylvania State University, University Park, PA 16802.
†Present address: Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138.

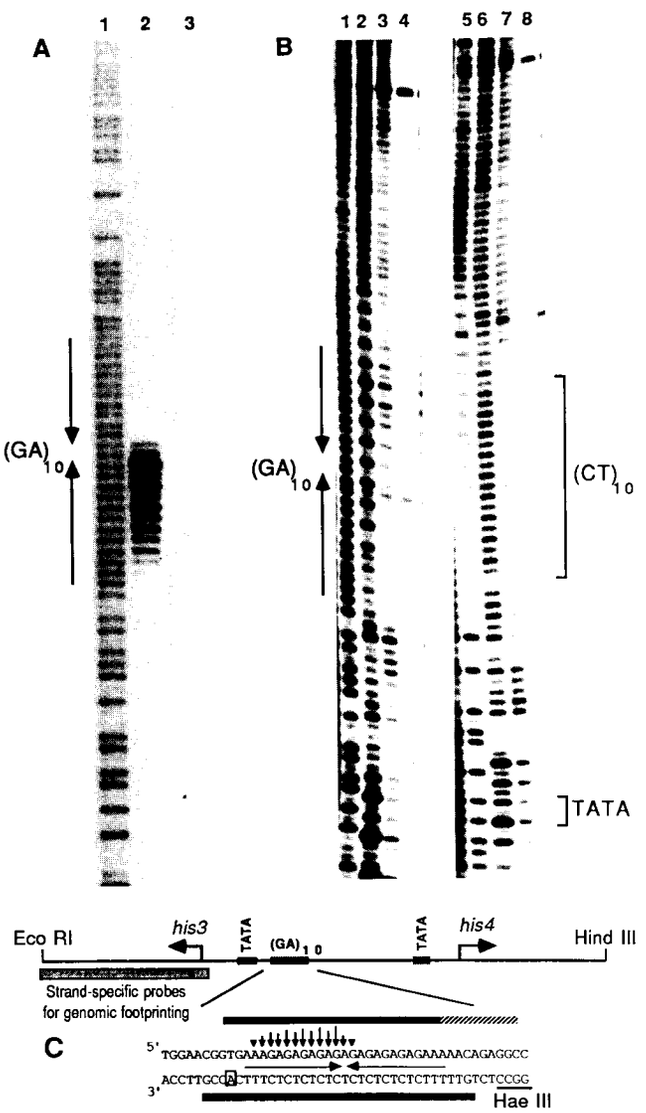

Fig. 1. The CT sequence in the intergenic region between the *his3* and *his4* promoters deforms in supercoiled plasmids at low *p*H and is protected from digestion by DNase I in nuclei. (**A**) Detection of S1 nuclease–sensitive sites in a supercoiled plasmid containing the *his3* and *his4* promoters (*25*). Lane 1, the products of a partial purine cleavage reaction; lane 2, the products of digestion with S1 nuclease of a supercoiled plasmid at *p*H 5.5; lane 3, a sample treated identically to that in lane 2 with the exception that S1 nuclease was not added. (**B**) Genomic footprinting analysis of the region containing the *his3* and *his4* promoters (*26*). Lanes 1 to 4, detection of cleavages on one strand; lanes 5 to 8, cleavages on the complementary strand. Lanes 1 and 5, the products of partial cleavage at purine residues in purified genomic DNA; lanes 2 and 6, the products of partial digestion by DNase I of purified genomic DNA; lanes 3 and 7, the products of partial digestion with DNase I of DNA in isolated nuclei; lanes 4 and 8, genomic DNA isolated from nuclei that were incubated in DNase I digestion buffer without DNase I (a control for endogenous nuclease activity). (**C**) The overall organization of the *his3-his4* promoter region. The transcription initiation sites are indicated by the arrows. The respective TATA boxes of each histone gene and the long stretch of alternating C and T residues are shown. Strand-specific probes used in (B) are homologous to the indicated region in *his3*. Below the restriction map is the sequence of the region encompassing the CT stretch. Genomic footprints are shown on the respective strands by the bold lines; the hashed lines represent uncertainty in delimiting the ends of the footprints. Vertical arrows mark the sites of S1 cutting in supercoiled plasmids at *p*H 5.5; their sizes reflect the relative efficiency of cutting at each nucleotide. Horizontal arrows delineate the mirror repeat. The Hae III restriction site and the boxed A residue are relevant to the protocol for selective labeling of the CT region [see Fig. 2C and (*12*)].

(Fig. 1B, lane 3). The protection was apparent even after digestion with ten times the amount of DNase I used for the experiment shown in Fig. 1B (*8*). As a torsionally strained region of DNA would not persist under these more extensive digestion conditions, it is likely that the protection was produced by a bound protein and not by a superhelical-dependent deformation in the DNA double helix. Protection was also seen on the TATA sequence located upstream of the *his3* transcription start and was presumably produced by a TATA factor (Fig. 1B).

The pattern of cleavage observed for the *his3* in nuclei is the product of approximately 100 copies of the histone gene cluster (*9*). The sequence composition of these copies must be homogeneous, because the pattern produced for the region from the restriction site to the proximal side of the CT sequence exactly matches that expected of a cloned representative of the region (compare Fig. 1A, lane 1, and Fig. 1B, lane 1). The cleavage pattern on the distal side of the CT region is less distinctive. However, a similar indirect end-labeling analysis from the opposite side of the CT sequence shows a precise pattern of purine cleavage sites up to the CT sequence (*8*). Thus, the imprecision (Fig. 1B) is due to the CT region being slightly heterogeneous in length.

Proteins that interact with the CT sequence (*10*) were isolated from nuclear extracts of *Drosophila* embryos (*11*) by affinity chromatography. Interaction of the affinity-purified protein fraction with the intergenic region located between the *his3* and *his4* promoters occurred on linear DNA fragments (Fig. 2A, lanes 1 and 6), indicating that DNA supercoiling was not required. In addition to the protection that occurred on the region of alternating C and T residues upstream of *H3*, there was an interaction of protein with a region containing the nucleotide sequence GAGAGAG upstream of *his4*, its complementary sequence on the opposite strand being CTCTCTC.

The final step in the purification of the CT-binding protein was DNA affinity chromatography on a column consisting of multimers of the annealed oligonucleotides 5′-AGAGAGAGAGAGAGAAAAGAGAGAG-3′ and 3′-CTCTCTCTCTCTTTTC-TCTCTCTCT-5′. Fractions from this column exhibiting binding activity were subjected to SDS–polyacrylamide gel electrophoresis, which revealed several polypeptides between 66 and 105 kD (Fig. 2B). Successive passages of the protein mixture over the DNA affinity column, even in the presence of nonspecific carrier DNA [poly(dI-dC)], did not significantly alter the polypeptide composition (*8*).

To identify which polypeptide interacts with the CT sequence upstream of *his3*, we used a protein-DNA cross-linking analysis (*12*). A 66-kD protein became cross-linked to the pyrimidine strand of the CT region (Fig. 2C). The labeling of this protein was sequence-specific, as the corresponding nonradioactive polymer inhibited labeling (Fig. 2C, lane 3), whereas a polymer composed of the minimal heat shock element (HSE) (5′-CTAGAAGCTT-3′/3′-TTCGAAGATC-5′)$_n$ did not (Fig. 2C, lane 2). The latter sequence does not contain a region of alternating C and T residues of more than three bases. To ensure that the analysis focused only on interactions with the CT region, we selectively labeled DNA fragments in the pyrimidine strand of the CT sequence (*12*).

The *hsp70* and *hsp26* heat shock gene promoters also include potentially deformable DNA sequences, as demonstrated by their sensitivity to S1 nuclease in supercoiled plasmids at low *p*H (*13, 14*). We thus determined whether our protein preparation bound to these sequences. DNase I footprinting analyses indicated that protein bound to the potentially deformable sequences upstream of the *hsp26* and *hsp70* genes (Fig. 3, lanes 1, 6, 7, and 12). In addition, binding occurred on two short CT sequences that are interdigitated with the HSEs of *hsp70* (*15*).

Two types of evidence suggest that the same or closely related proteins are responsible for all of the interactions of the affinity-purified proteins with the histone and heat shock gene promoter regions (Fig. 4). First, the proteins responsible were eluted from a Mono S column at a defined point in a salt gradient (*8*). Second, the interactions that occur within the heat shock gene promoters are sensitive to competition by the CT-GA oligomer but not by the oligomer of the

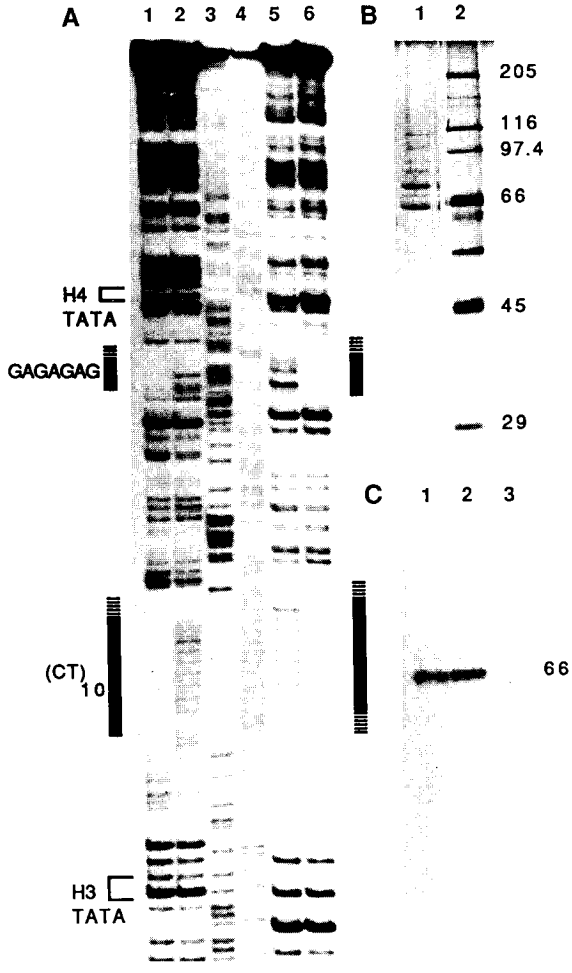

Fig. 2. DNA binding and polypeptide composition of the affinity-purified CT-binding proteins. (**A**) DNase I footprinting analysis of the CT-binding protein on the *his3-his4* promoter region (*27*). Lanes 1 to 3, the products of cleavages on one strand labeled on the 3′ end with reverse transcriptase; lanes 4 to 6, the products of cleavages on the complementary strand labeled on the 5′ end with polynucleotide kinase. Lanes 1, 2, 5, and 6, the products of digestions with DNase I; samples in lanes 1 and 6 were produced in the presence of the CT-binding proteins and samples in lanes 2 and 5 were produced without addition of the protein. The samples in lanes 3 and 4 were produced by partial cleavage at purine residues. Because of the position of the radioactivity on the two strands, the cleavage patterns in lanes 1 to 3 are two bases out of alignment with lanes 4 to 6. Footprints are shown on the respective strands by bold lines; the stippled bars represent uncertainty in delimiting the end of the footprints. (**B**) SDS-polyacrylamide gel analysis of the protein fraction from the DNA affinity column (*10*). Lane 1 contains 10 μl of the affinity-purified protein (*10*). Lane 2 contains molecular mass markers (approximately 30 ng of each protein; sizes shown in kilodaltons). After electrophoresis, the proteins were detected by staining with silver. (**C**) Protein DNA cross-linking analysis of the affinity-purified, CT-binding fraction. The affinity-purified fraction was incubated as in (A) with a DNA fragment that had been selectively labeled with ^{32}P on the pyrimidine strand in the region of the CT sequence, and cross-linking analysis was then performed (*12*). An autoradiogram of a dried SDS-polyacrylamide gel containing the radioactively tagged proteins is shown. Lane 1, proteins tagged in the absence of any synthetic oligonucleotide competitor; lanes 2 and 3, proteins tagged in the presence of a ligated polymer of the HSE (5′-CTAGAAGCTT-3′/3′-TTCGAAGATC-5′)$_n$ or in the presence of a ligated polymer of the CT sequence (5′-AGAGAGAGAGAGAGAAAAGAGAGAG-3′/3′-CTCTCTC-TCTCTTTTCTCTCTCTCT-5′)$_n$, respectively. The position of a 66-kD band is shown.

minimal HSE (*8*), as shown earlier for the *his3-his4* sequences; the failure of the HSE sequence to compete indicates that the heat shock transcription factor does not contribute to the footprints observed in Fig. 3.

The CT-binding protein that we have affinity-purified appears to be the same as or closely related to the previously described GAGA factor (*16*); the two proteins are both 66 kD, and purified GAGA factor produces DNase I footprints on the histone CT regions that are identical to those obtained with our protein preparations (*8*). The GAGA factor was isolated from a DNA affinity column composed of (5′-GATCCTGGCTCTCTGTTTC-3′/3′-GACCGAGAGACAAAGCTAG-5′)$_n$ and was found to interact with short CT sequences that are interdigitated with the binding sites of a second DNA-binding protein produced by the *zeste* gene (*17*). Both the *zeste* gene product and the GAGA factor stimulate in vitro transcription from the *Ubx* promoter, indicating that both are putative transcription factors.

We propose that the proteins interacting with CT sequences might participate in either the assembly or the maintenance of a promoter structure poised for transcription. The strength of the genomic footprint that we detected upstream of the *his3* promoter (Fig. 1B) indicates that most of the *his3* genes are associated with this CT-binding protein. Most of these genes are inactive, as only a small fraction of the cells in 6- to 18-hour-old embryos would be in the S phase of the cell cycle, which is when the histone genes are expressed (*18*). The inactive histone genes might be maintained in the poised state by association with the CT-binding proteins. A similar role can be proposed for the CT-binding protein interacting with the heat shock genes. The heat shock genes are not expressed in normally growing cells; they are rapidly and dramatically induced by various types of stress (*19*). Nonetheless, both the TATA factor and RNA polymerase II are associated with their promoter regions before transcriptional activation (*7, 20–22*). Genomic footprinting analyses (similar to those in Fig. 1B) indicate that the CT sequence upstream of *hsp26* is resistant to cleavage by DNase I in nuclei from non–heat shocked embryos (*7*). Since this resistance is an extension of a much larger DNase I–resistant region produced by a nucleosome, it was unclear in the earlier analysis whether a distinct protein was associated with the CT sequence. By analogy to the histone region, this seems likely to be the case.

Both large and small CT sequences located upstream of *hsp70* interact with our affinity-purified protein. Deletion analyses of *hsp70* indicate that the large CT regions are not required for heat shock induction (*23, 24*). However, the function of the CT sequences that are interdigitated with the HSE has not been systematically analyzed.

The potential for CT sequences to deform from a normal double-helical conformation has led to the proposal that the deformed structure of these sequences may function in gene expression (*5*). Although we do not rule out the transient formation of such structures, our results provide support for the hypothesis that CT sequences mediate their effects on transcription by interacting with sequence-specific DNA-binding proteins.

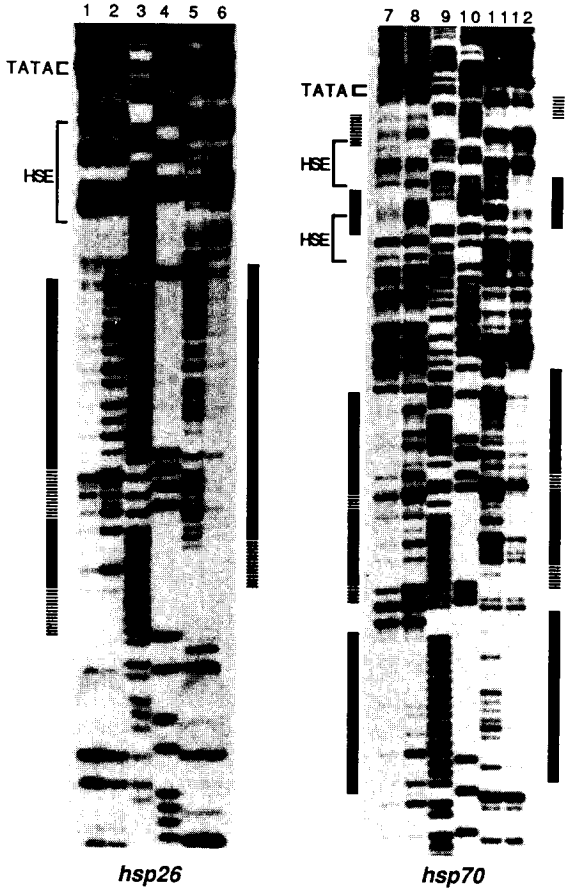

Fig. 3. DNase I footprinting analyses of the affinity-purified, CT-binding proteins on the *hsp26* and *hsp70* heat shock gene promoters. Binding analyses on the *hsp26* and *hsp70* promoter fragments were performed as described (*27*). The *hsp26* DNA (lanes 1 to 3) and the *hsp70* DNA (lanes 7 to 9) are the products of cleavages on one strand labeled at the 3′ end with reverse transcriptase; the corresponding *hsp26* DNA samples (lanes 4 to 6) and the *hsp70* samples (lanes 10 to 12) are the products of cleavages on the complementary strand labeled at the 5′ end with polynucleotide kinase. Lanes 1, 2, 5, 6, 7, 8, 11, and 12 are the products of DNase I digestions; samples in lanes 1, 6, 7, and 12 were produced in the presence of the CT-binding proteins; and samples in lanes 2, 5, 8, and 11 were produced without the addition of protein. The samples in lanes 3, 4, 9, and 10 were produced by partial cleavage at purine residues. Because of the position of the radioactivity on the two strands, the cleavage pattern on one strand is slightly out of alignment with its complement. Footprints are shown on the respective strands by bold lines; the stippled bars represent uncertainty in delimiting the ends of the footprints.

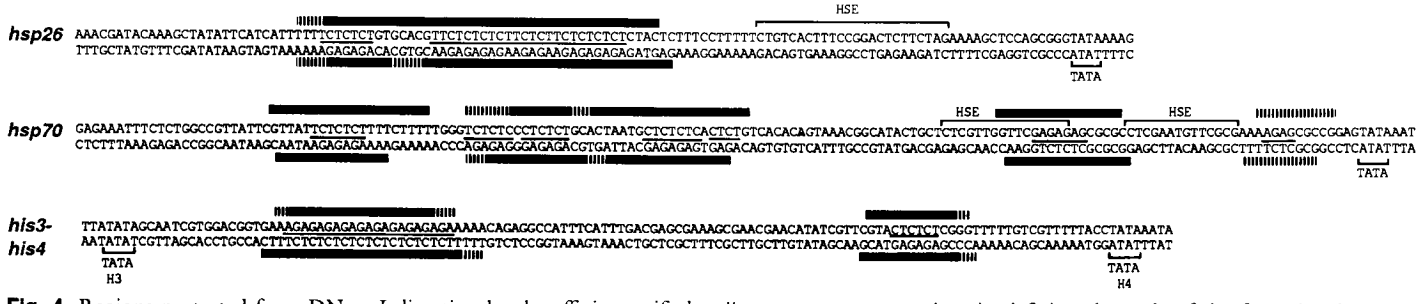

Fig. 4. Regions protected from DNase I digestion by the affinity-purified CT-binding proteins. Sequences of the complementary DNA strands of the *hsp26*, *hsp70*, and *his3-his4* promoter regions are shown. The DNase I footprints on each strand are shown by the bold lines located above and below the respective sequences; the stippled regions at the ends of some bold lines represent uncertainty in defining the ends of the footprint due to a paucity of DNase I cuts in the region. The CT sequences within each footprint are underlined. The heat shock regulatory sequences (HSEs) are bracketed above the sequences of each heat shock promoter; the TATA elements are bracketed below each sequence.

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10. The CT-binding protein was detected by DNase I footprinting analysis on a histone *his3-his4* promoter fragment (*27*). For purification of the CT-binding proteins, an ammonium sulfate precipitate of a nuclear extract was prepared (*11*) from 150 g of frozen *Drosophila* embryos that had not been heat-shocked. The precipitate was dissolved in 5 ml of a solution containing 0.1*M* KCl, 10% glycerol, and HEDP [25 m*M* Hepes (*p*H 7.6), 0.1 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT), and 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF)] and passed through a Sephacryl S300 gel filtration column (1.5 by 90 cm; Pharmacia) at a flow rate of 20 ml/hour. Virtually all of the CT-binding activity eluted in the first three 6.5-ml fractions that contained protein. These fractions were pooled and one third of the pooled material was applied to a Mono S column (Pharmacia). Proteins were eluted at a flow rate of 1 ml/min with a 20-ml gradient of 0.1 to 0.8*M* KCl in 0.1% NP-40, 10% glycerol, and HEDP. CT-binding activity eluted in two fractions between 0.32 and 0.38*M* KCl. The pooled fractions containing activity were adjusted to 0.3*M* KCl, 0.1% NP-40, poly(dI-dC) (50 μg/ml), 10% glycerol, and HEDP, and applied to a 2-ml DNA-affinity column at a flow rate of approximately 1 ml/hour. The column was produced essentially as described (*28*) and contained ligated oligomers of the sequence 5′-AGAGAGAGAGAGAGAAAAGAGAGAG-3′/3′-CTCTCTCTCTCTTTTCTCTCTCTCT-5′. Bound protein was eluted with 1-ml applications of 0.4 to 1*M* KCl in increments of 0.1*M* KCl in HEDP, 0.1% NP-40, and 15% glycerol. Fractions of 0.6 to 0.8*M* KCl contained the peak of binding activity, and these fractions were pooled, diluted to 0.3*M* KCl, HEDP, 0.1% NP-40, 15% glycerol, and poly(dI-dC) (5 μg/ml), and applied to a 200-μl DNA-affinity column at a flow rate of 1.25 ml/hour. Bound protein was eluted with consecutive 100-μl portions of 0.7*M* KCl, 0.1% NP-40, 15% glycerol, and HEDP. Virtually all of the activity was recovered in two fractions.
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12. Selective labeling was achieved by annealing a mixture of single-stranded *his3-his4* DNA containing the purine strand of the CT region and an isolated Hae III–Hind III restriction fragment from the H3/H4 S/X clone (*11*). The Hae III end is immediately adjacent to the CT stretch (Fig. 1C). Extension by DNA polymerase in the presence of radioactive thymidine triphosphate (TTP) and nonradioactive deoxycytidine triphosphate (dCTP) and deoxyguanosine triphosphate (dGTP), but in the absence of deoxyadenosine triphosphate (dATP), results in incorporation of radioactive thymidine throughout the polypyrimidine region but termination at the adjacent adenine (boxed A in Fig. 1C). The subsequent addition of all four nonradioactive nucleotide triphosphates at 400 μ*M* resulted in essentially selective labeling of the polypyrimidine strand in the CT region. A Xho I–Bam HI restriction fragment (450 bp) was then isolated and used in the cross-linking analysis. The Xho I and Bam HI restriction sites are adjacent to the Eco RI and Hind III sites shown in Fig. 1. Approximately 75,000 cpm were used in each binding reaction. The sample was irradiated for 2 min with a shortwave ultraviolet light provided by an inverted transilluminator (*29*). Noncovalently linked proteins were dissociated from the DNA by adding 25 μl of 0.4% sarkosyl and 2 m*M* PMSF, followed by 50 μl of 1% NP-40, 10 m*M* $MgCl_2$, 5 m*M* $CaCl_2$, and 20 m*M* tris-HCl (*p*H 8.0) to render the samples suitable for digestion with nucleases (*29*). The samples were digested for 1 hour at 37°C with 5 units of DNase I and 3 units of micrococcal nuclease, precipitated with trichloroacetic acid at a final concentration of 20%, washed with acetone, and then subjected to electrophoresis on a 10% SDS-polyacrylamide gel.
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25. The histone promoter–containing plasmid was made by cloning the insert of H3/H4 S/X (*11*) into M13mp18. For the sample in lane 2 of Fig. 1, 15 μg of supercoiled plasmid (double-stranded replicative form) was treated in 240 μl of a solution containing 200 m*M* NaCl, 50 m*M* 2-[*N*-morpholino]ethane sulfonic acid (*p*H 5.5), 1 m*M* $ZnSO_4$, 0.5% glycerol, and 48 units of S1 nuclease for 10 min at 25°C. The DNA was purified, digested with Bst NI, denatured, and then 10 ng was separated by electrophoresis on an 80-cm sequencing gel. Electrophoretic transfer and indirect end-labeling analysis with a strand-specific probe was performed as described (*14*). The hybridization probe was prepared by extension with the Klenow DNA polymerase fragment of the "hybridization" primer (New England Biolabs) annealed to a single-stranded M13 DNA template.
26. The DNA samples described by Thomas and Elgin (*7*), and prepared by treatment with DNase I, of either nuclei (digested with 2 units of DNase I per milliliter) or purified genomic DNA were digested with Taq I and processed on an 80-cm sequencing gel. After electrophoretic transfer, the histone sequences were detected with hybridization probes specific for either strand. These probes were produced (*25*) by extending primers annealed to single-stranded M13 clones containing the *his3-his4* promoter region. The radioactive deoxynucleotides had specific activities of 3000 Ci/mmol.
27. For DNase I footprinting reactions, 3 μl of affinity-purified DNA-binding protein was incubated for 20 min at 25°C in a 25-μl reaction mixture containing 500 ng of Hae III–digested *Escherichia coli* DNA, 80 m*M* KCl, 10 m*M* Hepes (*p*H 7.6), 5 m*M* $MgCl_2$, 0.5 m*M* DTT, 10% glycerol, 0.1% NP-40, and ~0.2 ng of end-labeled DNA. DNase I (0.05 unit) was added and the sample was incubated for an additional 35 s at 25°C. The reaction was quenched with 25 μl of 1% sarkosyl, 15 m*M* EDTA, and proteinase K (0.1 μg/μl), and the DNA was then purified by extraction with phenol and precipitation with ethanol. DNA samples were analyzed on 8% sequencing gels.
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30. The purified GAGA factor was provided by M. Biggin. Supported by NSF grant DCB-8601449 and NIH grant GM31532 to S.C.R.E. and PHS postdoctoral fellowship F32 GM107982 from the NIH to D.S.G.

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Identification of Monocyte Chemotactic Activity Produced by Malignant Cells

D. T. GRAVES,* Y. L. JIANG, M. J. WILLIAMSON, A. J. VALENTE

Human malignant cells secrete low molecular size proteins that attract peripheral blood monocytes and may be responsible for the accumulation of tumor-associated macrophages observed in vivo. Similar chemotactic proteins are secreted by cultured vascular smooth muscle cells. The predominant monocyte chemoattractants produced by tumor cells of differing origin were demonstrated to be related to smooth muscle cell–derived chemotactic factor. Thus, a single class of chemotactic proteins is produced by different cell types, which suggests a common mechanism for the recruitment of monocytes and macrophages. These results are significant in view of the potential of macrophages to affect tumor growth.

A CHARACTERISTIC FEATURE OF MACrophages is their ability to infiltrate tumors. The cellular mass of a solid tumor can consist of up to 80% macrophages (*1*). Macrophages may stimulate tumor growth through the production of proliferative and angiogenic factors (*2*) or exert an inhibitory effect through the production of factors such as interferon, tumor necrosis factor, and transforming growth factor–β (*3, 4*). The macrophage content of tumors is sustained primarily through the recruitment of circulating monocytes, which undergo maturation in situ to macrophages (*5*). Formerly, it was thought that such recruitment resulted from immune recognition of neoplastic cells (*6*). This now appears unlikely, however, as there is little correlation between immunogenicity and the level of tumor-associated macrophages (*7*).

D. T. Graves, Y. L. Jiang, M. J. Williamson, Department of Oral Biology, Boston University Medical Center, Boston, MA 02118.
A. J. Valente, Cleveland Research Institute, Cleveland, OH 44116.

*To whom correspondence should be addressed.