Sequence-specific Transcriptional Antirepression of the Drosophila Krüppel Gene by the GAGA Factor*

(Received for publication, July 23, 1990)

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We have analyzed the proximal promoter of the Drosophila Krüppel (Kr) gene. A 44-base pair fragment containing the RNA start sites contains significant promoter activity, and this minimal promoter is flanked both upstream and downstream by binding sites for the GAGA factor. The GAGA factor is the predominant sequence-specific DNA binding factor that interacts with the Kr promoter region, and the purified protein activates Kr transcription in vitro. However, strong transcriptional activation of Kr as well as of Ultrabithorax, another GAGA factor-responsive gene, requires the presence of a DNA binding transcriptional repressor. The GAGA factor is able to relieve this repression in a binding site-dependent manner, and, thus, these data suggest that the GAGA factor functions as an antirepressor, rather than an activator, of the Kr gene.

Although there are a variety of means by which the expression of genes can be regulated, this process commonly occurs at the level of transcription initiation. Synthesis of mRNA is carried out by RNA polymerase II and several auxiliary factors (for recent review, see Saltzman and Weinmann, 1989), and the activity of these enzymes can be modulated by other factors, some of which are sequence-specific DNA binding proteins. These DNA binding proteins are often expressed in a spatially and temporally restricted manner, and they appear to be important components in gene-specific transcriptional regulation (for a recent review, see Mitchell and Tjian, 1989). Yet, to elucidate the means by which the sequence-specific factors control expression of genes, it is first necessary to understand the function of the regulatory factors within the framework of the basic mechanism of transcription. Thus, studies on both the sequence-specific factors and the RNA polymerase II transcriptional apparatus are essential for providing information on interactions between the DNA binding factors and the polymerase machinery.

We have initiated a biochemical analysis of RNA polymerase II transcription in Drosophila with the aim of studying the mechanism of eukaryotic transcription regulation at the molecular level (for a recent review of Drosophila transcription factors, see Biggin and Tjian, 1989). To this end, we have fractionated RNA polymerase II and the auxiliary factors from Drosophila embryos (Wampler et al., 1990) and examined the kinetics and mechanism of Drosophila RNA polymerase II transcription (Kadonaga, 1990). In these studies, we have chosen to focus upon transcription of the Drosophila segmentation gene Krüppel (Kr; for review, see Gaul and Jackle, 1987). Kr is a member of the Drosophila gap genes, which are involved in the early steps in the formation of anteroposterior boundaries in the embryo (for recent reviews on Drosophila segmentation, see Akam, 1987; Ingham, 1988; Scott and Carroll, 1987). In the syncytial blastoderm, Kr is expressed in a circumferential band that spans from roughly 45 to 55% of the egg length from the posterior pole (Knipple et al., 1985), and Kr transcripts are mainly detectable in 2- to 5-h-old embryos (Rosenberg et al., 1986). In addition, Kr encodes a protein containing zinc fingers (Rosenberg et al., 1986) that is a sequence-specific DNA binding protein (Pankratz et al., 1989; Stanoevic et al., 1989; Treisman and Desplan, 1989) and presumably a transcription factor that regulates other genes involved in the development of the embryo. Hence, we felt that Kr is an important gene to study because of its key position in the segmentation of the embryo. Furthermore, the Drosophila embryo in vitro transcription system is ideally suited for the examination of both the spatial and the temporal transcriptional control of the Kr gene.

As a first step in the transcriptional analysis of the Krüppel gene, we focussed upon the proximal promoter region that encompasses positions −861 to +426 relative to the major upstream RNA start site. At present, transcription that is mediated by factors acting within a few hundred nucleotides of the RNA start site can usually be reconstructed in vitro. In contrast, however, long range regulation that occurs from greater distances, such as the action of enhancers, generally cannot be accurately recreated in a biochemical reaction. For these reasons, in addition to the central importance of promoters in the transcriptional control of genes, we have chosen to examine initially the region immediately surrounding the Kr transcription start sites.

In this paper, we delineate segments of the Krüppel promoter that are important for transcription in vitro as well as purify and characterize the predominant sequence-specific DNA binding protein that interacts with this region. We find that this protein, which had been previously characterized as the GAGA factor (Biggin and Tjian, 1988), increases Kr transcription by interactions with binding sites that are downstream of the RNA start sites. Moreover, the GAGA factor appears to function as an transcriptional antirepressor, rather than a true activator, of the Kr gene.
EXPERIMENTAL PROCEDURES

Materials—Ribonucleoside 5'-triphosphates and deoxyribonucleoside 5'-triphosphates were purchased from Pharmacia LKB Biotechnology Inc. (ultrapure fast protein liquid chromatography-purified grade) from Life Sciences. Kinetochorinycin D, and Hepes were obtained from Sigma. Protease K was from Boehringer Mannheim. DNase I (DFPF grade) was purchased from Worthington. Exonucleolase III and mung bean nuclease were from Stratagene. Carrier yeast RNA was prepared by exhaustive extraction of crude TruZol yeast RNA (Sigma) with phenol and then with chloroform followed by dialysis to remove traces of residual chloroform. Synthetic oligonucleotides were prepared by Operon Technologies. Inc. Chromatography resins were obtained from Pharmacia.

Synthetic Oligonucleotides—The primer for reverse transcription analysis of the Kruppel RNA, named Kr5, is 5'-TATTACCTGGGTTGGTTGACACAC-3', and hybridizes to the Kr template from +45 to +72 relative to the major upstream start site. The primer for reverse transcription analysis of adenovirus E4 RNA, named AdE4, is 5'-CCTTACGACGCACTAAGCT-3', and hybridizes to the adenovirus E4 template from +78 to +97 relative to the major upstream start site. Other synthetic oligonucleotides used in this work are the following: Kr5, 5'-GAGTCCTTTGTATCAGTCGTTGTGACACAC-3', which hybridizes to pUC plasmids downstream of the polylinker and has the opposite sense of the lacZ' mRNA (similar to the M13 universal primer); M13RS, 5'-AACAGCTTACAGCATGTTGCAGCT-3', which hybridizes to pUC plasmids upstream of the polylinker and has the same sense as the lacZ' mRNA (similar to the M13 reverse sequencing primer); and the Ubx primer, a gift of M. Biggin, which has been described previously (Biggin and Tjian, 1988). Other synthetic oligonucleotides used in this work are the following: Kr5, 5'-GAGTCCTTTGTATCAGTCGTTGTGACACAC-3', which hybridizes to Kr and contains Kr sequences from -31 to +10 relative to the major upstream RNA start site; Kr6, 5'-GAGTCCTTTGTATCAGTCGTTGTGACACAC-3', which hybridizes to Kr and contains Kr sequences from -27 to +13 relative to the RNA start site; Kr7, 5'-GAGTCCTTTGTATCAGTCGTTGTGACACAC-3', which hybridizes to Kr and contains Kr sequences from -4 to +10 relative to the RNA start site; and Kr8, 5'-GAGTCCTTTGTATCAGTCGTTGTGACACAC-3', which is complementary to Kr7 and contains Kr sequences from -4 to +13 relative to the transcription start site.

Plasmids—Kruppel DNA was obtained from Michael Hoch and Dr. Herbert Jache (University of Munich) as pHISX, which contains a 1.3-kb SalI to XbaI fragment of the Kr gene that encompasses the region from -861 to +426 relative to the transcription start site (Rosenberg et al., 1986). This 1.3-kb SalI-XbaI fragment was subcloned into SalI and XbaI sites in the polylinker of pUC119 to give pKr, which was used in most of the in vitro transcription experiments. The Kr deletion mutants were prepared by digestion of pKr with HindIII, progressive deletion of DNA with exonuclease III and mung bean nuclease, digestion with EcoRI, and then ligation of gel-purified Kr fragments with pUC118 that was digested with Smal and EcoRI. The 3' deletion mutants were prepared by digestion of pKr with EcoRI, progressive deletion of DNA with exonuclease III and mung bean nuclease, digestion with HindIII, and then ligation of gel-purified Kr fragments with pUC118 that was digested with Smal and HindIII. Transcripts that were synthesized from the pKr which was also designated as either 5' -861 or 3' +426 in this manuscript, the 5' deletion mutants, and pKr3' +171 were analyzed with the Kr primer. Transcripts that were generated from pKr3' +19, pKr3' +9, pKr3' +2, and pKr3' -8 were analyzed with the JK4 primer. pKr -31/+131(118) and pKr -31/+131(119), which contain Kr promoter sequences from -31 to +131 relative to the major upstream start site, were constructed by insertion of the complementary oligonucleotides, Kr3' and Kr6, into either pUC118 or pUC19 digested with Asp-718 and BamHI. Transcripts synthesized from pKr -31/+131(118) and pKr -4/+131(118) were analyzed with the JK4 primer and yielded a 78-nucleotide extension product. Transcripts generated from pKr -31/+131(119) and pKr -4/+131(119) were analyzed with the M13RS primer and gave a 69-nucleotide extension product. pGEX4T and pGLOBE (pE4A-38) (Lin et al., 1988) were the gifts of M. Carey (Harvard University). pUbS5' -175 and pUbS5' -31 (Biggin and Tjian, 1988) were the gifts of M. Biggin (Yale University). All plasmids were subjected by two cycles of CsCl equilibrium gradient centrifugation and extracted with phenol and chloroform to remove contaminants. The identity of every plasmid preparation was confirmed by Southern blot analysis of the plasmid DNA using 5'32P-labeled primers and the Sequenase protocol (United States Biochemical Corp.).

Preparation of Protein Fractions—Drosophila embryo extracts were prepared with Canton S embryos collected between 0 and 12 h after fertilization that were stored for up to 3 days at 4 °C (Biggin and Tjian, 1988). Extracts were prepared from embryos that were indistinguishable from those that were prepared with embryos stored at 4 °C for 3 days or less. SK extracts were prepared by the method of Soeller et al. (1988), except that the nuclei wash step was omitted and the dialysis step was replaced by desalting with Sephadex G-25 SF. HP extracts were prepared according to the procedure of Heiermann and Pongs (1985) using 0.45 M KC1 in the nuclear extraction step. Cytoplasmic extracts were prepared as described by Biggin and Tjian (1988). The 0–50 mm ammonium sulfate fractions of the HP extract were prepared as follows (all operations were performed at 4 °C). Solid (NH4)2SO4 (0.314 g/ml of extract) was added to the crude HP extract to give a final concentration of 2 m (50% saturation). The crude extract was mixed or a short period of time with 4 M ammonium sulfate and then centrifuged at 15,000 rpm for 20 min. The pellet was suspended in 25 mm Hepes (pH 7.6), containing 100 mm KCl, 12.5 mm MgCl2, 0.1 mm EDTA, 1 mm dihydroethanol, 1 mm sodium bisulfite, 1 mm benzamidine, 0.1 mm phenylmethylsulfonyl-fluoride, and 10% (v/v) glycerol (0.17 ml of buffer per mg of original crude extract) and dialyzed against the same buffer until the conductivity of the sample was equal to that of the buffer. The 0–50% fraction was then clarified by centrifugation in a Sorvall SS-34 rotor at 10,000 rpm for 10 min. The HP factor was purified from Drosophila embryos according to the procedure of Biggin and Tjian (1988), except that the heparin-agarose and Sephacryl S-300 SF chromatography steps were replaced by Sephacryl S-400 HR size exclusion chromatography. The GAGA factor-depleted extracts were prepared by combining 1 ml of extract with 0.2 ml of GAGA factor DNA affinity resin in a 1.5 ml microcentrifuge tube, mixing the tube for 30 to 60 min on a rotating wheel at 4 °C, and then removing the DNA affinity resin by a 10,000 rpm spin for 10 min. The GAGA factor was then pelleted in a Sorvall SS-34 rotor at 10,000 rpm for 10 min. The GAGA factor was purified to homogeneity using bovine y-globulin as the reference.

In Vitro Transcription and DNase I Footprinting—In vitro transcription reactions and primer extension analysis of the RNA was carried out as described previously (Kadonaga, 1990). Reactions that included addition of purified GAGA factor were performed as follows: the GAGA factor was fractionated with thetemplate DNA at 2°C (on ice) for 10 min; the transcription extract and ribonucleoside 5'-triphosphate (at 2 °C) were added to the GAGA factor template DNA mixture on ice; and then the complete reaction mixtures were incubated at 21 °C in a water bath for 30 min. DNase I footprinting was performed as described by Dynan and Tjian (1983). All experiments were carried out a minimum of two independent times, and the relative levels of transcription were measured by using an LKB Ultrascan scanning densitometer.

RESULTS

The Kruppel Promoter Is Accurately Transcribed in Vitro

We first examined if the Kruppel gene can be transcribed accurately in vitro with extracts derived from Drosophila embryos. In these experiments, we used the plasmid pKr as the template DNA. This plasmid contains a 1.3-kb SalI to XbaI fragment of the Kr gene, which was provided by Michael Hoch and Dr. Herbert Jache, inserted into the SalI and XbaI sites in the polylinker of pUC119. As shown in Fig. 1, Kr transcription initiates at several sites over a 10-nucleotide

1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; kb, kilobase(s); bp, base pair(s).
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Fig. 1. The Drosophila Kruppel gene is transcribed accurately in vitro. Standard in vitro transcription reactions were carried out using the SK nuclear extract (150 µg of protein) with 0 µg/ml (lane 1), 4 µg/ml (lane 2), or 20 µg/ml (lanes 3–5) pKr template DNA. As controls, transcription extract was omitted from the reaction (lane 4), and 4 µg/ml α-amanitin was added to the reaction (lane 5). In vitro Kr transcripts from 1 µg of poly(A⁺) RNA from 4- to 6-h Drosophila embryos are shown in lane 6. The products of reverse transcription of the Kr RNA are designated by the bracket at the left. The DNA sequence of the Kr promoter region in the vicinity of the start sites was generated by plasmid DNA sequencing of pKr with the major RNA start sites are marked by the open circles. The major upstream start site is designated as +1. The sequence, 5' ATCAGTC, which conforms with the consensus for Drosophila RNA polymerase II promoters that was originally proposed by Hultmark et al. (1986), is underlined. For the DNA sequence of the Kr promoter region, see Fig. 4D.

stretch of the promoter, and the RNA synthesized in vitro using a crude embryo nuclear extract (Fig. 1, lanes 2 and 3) is indistinguishable by primer extension analysis from Kr transcripts in poly(A⁺) RNA obtained from 4- to 6-h embryos (Fig. 1, lane 6). Furthermore, transcription in vitro is sensitive to 4 µg/ml α-amanitin (Fig. 1, lane 4) and is dependent upon both the pKr template and the embryo extract (Fig. 1, lanes 1 and 5). As depicted at the bottom of Fig. 1, the major upstream RNA start site is designated as position +1 of the promoter (for the DNA sequence of the Kr proximal promoter region, see Fig. 2D). The +1 site is the major start site in vivo, whereas the +1 and +5 sites are used with comparable efficiency under standard conditions in vitro. Transcription initiation at +1 versus +5 depends upon the balance of ribonucleoside triphosphates in the reaction mixture. The +1 transcript begins 5' UAU . . . , and the +5 transcript begins 5' AGU . . . We had previously observed the preformed transcription initiation complexes initiate at +1 upon addition of UTP and ATP (which yields the trinucleotide, 5' UAU), whereas initiation complexes will begin at +5 in the presence of ATP and GTP (which yields the dinucleotide, 5' AG) (Kadonaga, 1990).

The location of the Kr transcription start sites is approximately 150 nucleotides upstream of the Kr start site that was previously reported by Rosenberg et al. (1986). We examined the Kr promoter region by primer extension analysis with two different primers that hybridize downstream of the previously reported start site and could not find any detectable transcript originating from that site in poly(A⁺) RNA from Drosophila embryos (data not shown). Moreover, we have confirmed our start sites with S1 protection experiments (data not shown), and, consequently, we are confident of the authenticity of the Kr RNA start sites that are reported here. Lastly, the transcription start sites of the Kr gene also contain the sequence, 5' ATCAGTC, which conforms with the consensus of 5'

2 C. Schröder and H. Jäckle, unpublished data.
ATCG/TTC/T for Drosophila start sites that was proposed by Hultmark et al. (1986).

Mapping of the Krüppel Minimal Promoter by Analysis of Deletion Mutants—To identify essential elements of the Kr promoter region, we generated and characterized a series of deletion mutants of the promoter. In this analysis, we employed nuclear extracts from Drosophila embryos that were prepared by two different procedures to examine if the different extracts would yield similar in vitro transcription data. The first embryo extract, which we designated as the "SK extract" (Soeller-Kornberg), was prepared by the procedure of Soeller et al. (1988), and the second extract, named the "HP extract" (Heiermann-Pongs), was prepared according to the method of Heiermann and Pongs (1985). These two procedures differ mainly in the conditions used for salt extraction of the nuclei as well as the use of an ammonium sulfate precipitation step in the SK extract but not the HP extract. We tested these two extracts with a series of deletion mutants of the Kr promoter and found that the different extracts gave generally similar results, which are summarized in Table 1. Using the SK extract, we observed a high level of transcription with 5' deletion mutants from -861 to -48 relative to the major upstream start site (except for the -141 mutant), a progressive decrease from -48 to -11, and no detectable transcription from the +15 mutant. In a parallel series of reactions with the HP extract, there was a more marked decrease of transcription from the 5' -861 to -48 deletion mutants. We also examined the transcription from 3' deletion mutants of the Kr proximal promoter with the SK and HP extracts, and the 3' +171, +19, and +9 mutants were transcribed at least 14% as efficiently as the reference 5' -861/3' +426 Kr promoter, whereas there was no detectable transcription from the 3' +9 and 3' -8 mutants. Finally, we tested the transcriptional properties of fragments of the Kr promoter that spanned positions 5' -31/3' +13 and 5' -4/3' +13. The 5' -31/3' +13 fragment contained roughly 15-20% of the activity of the reference 5' -861/3' +426 promoter, while there was no detectable transcription from the 5' -4/3' +13 fragment. Hence, we have reduced the essential components of the Kr proximal promoter to a 44-bp fragment from positions -31 to +13 of the major upstream RNA start site, and we will refer to this 44-bp fragment as the Kr minimal promoter.

The GAGA Factor Binds to the Krüppel Proximal Promoter—To identify factors that interact with the Kr promoter, we carried out a DNase I footprinting analysis of this region using crude nuclear extracts as well as partially purified protein fractions that were subjected to Sephacryl S-400 HR size exclusion chromatography and/or heparin-Sepharose CL-2B chromatography (data not shown). We observed extensive binding of factors throughout the Kr promoter with these protein fractions and noticed that many of the binding sites contained GAGA sequences. We thus suspected that at least some of the footprints were due to binding of the GAGA factor, a protein that was originally identified by Biggin and Tjian (1988) on the basis of its binding to the Drosophila Ultrabithorax (Ubx) promoter. We then purified the GAGA factor from Drosophila embryos to greater than 95% homogeneity (Fig. 2A) and tested the binding of the purified protein to the Kr promoter (Fig. 2, B and C). We found that all of the footprints that were previously observed in the crude or partially purified extracts were due to binding of the GAGA factor. The GAGA factor binds to three regions upstream of the RNA start sites and to three regions downstream of the start sites, as summarized in Fig. 2D.

The GAGA Factor Does Not Stimulate RNA Synthesis When Used in Conjunction with a "Cytoplasmic" Transcription Extract—Upon identification of the GAGA factor as the predominant DNA binding factor that interacts with the Kr promoter, we tested its ability to activate RNA synthesis. Biggin and Tjian (1988) had previously observed that the GAGA factor stimulates transcription of a Ubx template, named Ubx 5' -175, which contains three upstream GAGA binding sites, but that it does not affect transcription of a second Ubx deletion mutant, designated Ubx 5' -31, which does not possess any GAGA binding sites. In these experiments, the transcription reactions were carried out with a low salt extract of a Drosophila embryo homogenate termed "cytoplasmic fraction," which was devoid of GAGA factor activity that would interfere with the reconstituted reaction. Using an identically prepared cytoplasmic extract, we carried out in vitro transcription reactions of the Kr and Ubx (as a control) promoters in the presence and absence of the GAGA factor and did not observe transcriptional activation of either Kr or Ubx (Fig. 3). We were concerned about this discrepancy between our data and the previous results, and, consequently, we repeated the GAGA factor transcription experiments with three different preparations of the cytoplasmic extract and three different preparations of purified GAGA factor. In all of these experiments, we did not observe GAGA factor-mediated activation of either Kr or Ubx (data not shown). We then carried out a series of experiments to determine the basis of our inability to observe GAGA factor activation of Ubx, and we found that the cytoplasmic fraction contained high levels of nucleic acids that potently inhibited transcription in vitro. When we compared the levels of nucleic acids in the cytoplasmic fraction with that in the SK and HP extracts by

| Template | 5' End point | 3' End point | SK extract | HP extract |
|----------|--------------|--------------|------------|------------|
| pKr      | -861         | +426         | 100%       | 100%       |
| pKr5'-539| -539         | +426         | 122        | 89         |
| pKr5'-491| -491         | +426         | 136        | 81         |
| pKr5'-435| -435         | +426         | 98         | 76         |
| pKr5'-141| -141         | +426         | 45         | 52         |
| pKr5'-117| -117         | +426         | 98         | 63         |
| pKr5'-83 | -83          | +426         | 90         | 35         |
| pKr5'-70 | -70          | +426         | 70         | 38         |
| pKr5'-49 | -49          | +426         | 75         | 29         |
| pKr5'-48 | -48          | +426         | 82         | 18         |
| pKr5'-45 | -45          | +426         | 65         | 21         |
| pKr5'-26 | -26          | +426         | 27         | 7          |
| pKr5'-25 | -25          | +426         | 17         | 6          |
| pKr5'-11 | -11          | +426         | 11         | 4          |
| pKr5'+15 | +15          | +426         | 0          | 0          |
| pKr5'+171| +171         | +426         | 41         | 47         |
| pKr5'+19 | +19          | +426         | 22         | 14         |
| pKr5'+9  | +9           | +426         | 48         | 29         |
| pKr5'+2  | +2           | +426         | 0          | 0          |
| pKr5'-8  | -8           | +426         | 0          | 0          |
| pKr5'-31/+13(118) | -31   | +13         | 19         |            |
| pKr5'-31/+13(119) | -31   | +13         | 15         |            |
| pKr5'-4/+13(118)  | -4     | +13         | 0          |            |
| pKr5'-4/+13(119)  | -4     | +13         | 0          |            |
Transcriptional Antirepression by the GAGA Factor

In transcription experiments with the HP extract, the GAGA factor-depleted HP extract (Fig. 4) demonstrates decreased transcription of both Kr and Ubx genes compared to the HP extract. The GAGA factor acts in a binding site-dependent manner, as stimulation was observed only with the Kr and Ubx templates that contain GAGA binding sites (Fig. 4). The GAGA factor activates Kr transcription from its downstream binding sites, but not from the upstream binding sites. In addition, the GAGA factor weakly represses the Kr minimal promoter, which does not contain any GAGA factor recognition sequences. This observation of a promoter proximal transcription factor acting downstream of the RNA start sites was unexpected, and this finding is an unusual demonstration of activation by downstream promoter proximal factors in reconstituted transcription reactions with purified sequence-specific factors. However, in the control experiments with the Ubx promoter, we observe GAGA factor-mediated transcriptional activation from upstream binding sites. Consequently, the GAGA factor is apparently able to affect transcription from both upstream and downstream positions relative to the RNA start sites.

The GAGA Factor Functions as a Transcriptional Antirepressor of Krüppel—Why does the GAGA factor activate transcription better with the HP extract than the SK extract? Our data suggested a few possible explanations: 1) the SK extracts contain a GAGA factor inhibitor; 2) the GAGA factor derepresses the action of a general transcriptional inhibitor, such as a nonspecific DNA binding factor, that is present only in the HP extract; or 3) there is an auxiliary factor in the HP extract that mediates GAGA factor transcriptional activation. To examine if there is a factor (which could be either a repressor or activator) in the HP extract that interacts with the template DNA, we carried out a template commitment assay with two different GAGA factor-responsive promoters. As outlined in Fig. 5, template DNA1 (with or without GAGA factor) is preincubated with the HP extract, and this mixture is then combined with template DNA2 (with or without GAGA factor as with DNA1) and ribonucleoside triphosphates to initiate transcription. In this experiment, we found that transcription from template DNA1 was repressed relative to that of template DNA2 and that GAGA factor-mediated activation occurred only from the transcriptionally repressed template DNA1. Hence, the HP extract contains a transcriptional repressor, such as a nonspecific DNA binding factor (it inhibits both Kr and Ubx transcription), that interacts with DNA, and the GAGA factor appears to relieve repression by this inhibitory factor since GAGA factor-mediated activation was observed only with the repressed template DNA1.

To characterize the properties of the transcriptional repressor in the HP extract, we fractionated the basic RNA polymerase II transcriptional machinery from the inhibitory agarose gel electrophoresis, it was apparent that there were much higher levels of nucleic acids in the cytoplasmic extract. It is thus likely that the nucleic acids in the cytoplasmic fraction interfere with the interaction of the GAGA factor with DNA. We do not understand why we have not been able to reproduce GAGA activation with the cytoplasmic extract as observed previously by Biggin and Tjian (1988). There is only one apparent difference between our procedure for the preparation of the cytoplasmic extract and that of Biggin and Tjian. To disrupt the embryos, we used a Yamamoto LH-21 continuous homogenizer, whereas Biggin and Tjian (1988) employed a Potter-Elvejem homogenizer, and the processing of the embryos in the Yamamoto homogenizer may have resulted in higher levels of nucleic acids in the medium.

The GAGA Factor Stimulates Transcription of the Krüppel Gene from Its Downstream Binding Sites—It was apparent that the GAGA factor would not function in the transcription reaction with cytoplasmic fractions prepared in our laboratory, and, thus, we explored other means to examine the role of the GAGA factor in transcription of the Kr gene. In a previous study on the Drosophila Antennapedia P2 promoter, Perkins et al. (1988) had successfully depleted a SK nuclear extract of transcription factor DTF-1 by incubation of the extract with the DNA affinity resin that was used to purify the factor. In a similar manner, we depleted the GAGA factor from both the SK and HP nuclear extracts and then used the resulting GAGA factor-depleted extracts in reconstituted transcription reactions. GAGA factor-mediated transcriptional activation of both Kr and Ubx was observed with the GAGA factor-depleted HP extract (Fig. 4), but, in contrast, GAGA factor activation was either weak or not detectable with the GAGA factor-depleted SK extract (data not shown).

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To characterize the properties of the transcriptional repressor in the HP extract, we fractionated the basic RNA polymerase II transcriptional machinery from the inhibitory
factor. A major difference between the HP extract (in which GAGA factor activates transcription) and the SK extract (in which the GAGA factor marginally activates transcription) is that an ammonium sulfate precipitation (9–56% saturation) step is employed in the preparation of the SK extract but not the HP extract. Thus, we were able to prepare a fraction with RNA polymerase II transcriptional activity that was devoid of the transcriptional repressor by subjecting the HP extract to an ammonium sulfate precipitation (0–50% saturation). The HP extract (+repressor) and the 0–50% ammonium sulfate fraction (−repressor) were then characterized in parallel, as shown in Fig. 6. In these assays, transcription reactions were carried out under the following three conditions: 1) template DNA + extract only; 2) template DNA + extract + GAGA factor; or 3) template DNA + extract + pUC plasmid DNA (to compete with the template DNA for nonspecific DNA binding factors). With the HP extract, the GAGA factor activates transcription in a binding site-dependent manner (Fig. 6A, lanes 1, 2, 4, and 5), while pUC plasmid stimulates transcription nonspecifically, as it would be expected if pUC were competing with the template DNA for a DNA binding inhibitor of transcription (Fig. 6A, lanes 3, 4, and 6). However, in reactions carried out with the 0–50% ammonium sulfate fraction, neither GAGA factor nor pUC stimulates transcription (Fig. 6B, lanes 1–6). These data are all consistent with GAGA factor-mediated antirepression of a DNA binding transcriptional inhibitor that is present in the HP extract but not the 0–50% ammonium sulfate fraction.

Finally, we also examined the properties of a different sequence-specific transcriptional activator with the HP extract and the 0–50% ammonium sulfate fraction. In these experiments, we used a hybrid GAL4-VP16 protein that had been previously shown to bind to GAL4 recognition sequences and activate transcription in vitro (Chasman et al., 1989). The GAL4-VP16 fusion protein was used as a model protein for the class of transcription factors that contain acidic transcriptional activator domains (for review, see Ptashne, 1988). To test transcriptional activation by GAL4-VP16 in vitro, we used the previously characterized derivatives of the adenovirus E4 promoter that contained either 5 or 0 GAL4 binding sites upstream of the TATA box (Lin et al., 1988). By using the HP extract, GAL4-VP16 activates transcription in a binding site-dependent manner (Fig. 6A, lanes 7, 8, 10, and 11), whereas pUC stimulates transcription nonspecifically (Fig. 6A, lanes 7, 9, 10, and 12). However, in contrast to the GAGA factor, GAL4-VP16 activates transcription in a binding site-dependent manner with the 0–50% ammonium sulfate fraction (Fig. 6B, lanes 7, 8, 10, and 11). As expected pUC did not stimulate transcription with the 0–50% ammonium sulfate fraction (Fig. 6B, lanes 7, 9, 10, and 12). Hence, there is a clear distinction between transcriptional activation by the GAGA factor and the GAL4-VP16 fusion protein. Unlike the
GAGA factor, GAL4-VP16 is capable of transcriptional activation in the absence of the DNA binding repressor. Note, however, that the magnitude of transcriptional activation by GAL4-VP16 is consistently higher with the HP extract (8-fold stimulation) than with the 0-50% ammonium sulfate fraction (2.5-fold stimulation). Thus, in transcription reactions with the HP extract, the GAL4-VP16 protein is probably functioning both as an activator and antirepressor.

**DISCUSSION**

We have carried out an *in vitro* transcriptional analysis of the *Drosophila Krüppel* promoter. The minimal promoter can be reduced to a 44-bp element, and the GAGA factor is the predominant sequence-specific DNA binding protein that interacts with the flanking *Kr* promoter region. The GAGA factor stimulates transcription primarily through the downstream binding sites of the *Kr* gene, although it can function through the upstream binding sites of the *Ubx* gene. The GAGA factor derepresses the inhibitory action of a DNA binding factor that can be separated from the general RNA polymerase II transcription factors by precipitation with ammonium sulfate. Hence, the GAGA factor appears to be an antirepressor, rather than a true activator, of the *Kr* gene. These data are summarized in Fig. 7.

**Transcriptional Antirepression as a Mechanism for Gene Regulation**—In a general sense, transcriptional antirepression is an attractive mechanism for regulation of gene activity. According to this model, genes are repressed by nonspecific DNA binding factors in the ground state until they are derepressed (and perhaps also activated) by sequence-specific factors that bind to their promoter and enhancer regions. It is worthwhile to note that we have also observed a clear distinction between the properties of the GAGA factor and GAL4-VP16 activator, where the GAL4-VP16 protein, unlike the GAGA factor, was able to stimulate transcription in the absence of the transcriptional repressor. Hence, some sequence-specific DNA binding factors, such as the GAGA factor, may serve primarily as antirepressors, whereas other sequence-specific factors may resemble GAL4-VP16 and activate transcription beyond a basal, derepressed level.

It is also formally possible that transcriptional activation by the GAGA factor requires an auxiliary activator (i.e. both GAGA factor and the auxiliary activator are necessary for transcriptional activation) that is present in the HP extract but not the 0-50% ammonium sulfate fraction. It has been proposed that such auxiliary "co-activators" are necessary for transcriptional activation by Sp1 (Hoey et al., 1990; Pugh and Tjian, 1990; Peterson et al., 1990) and GAL4-VP16 (Berger et al., 1990; Kelleher et al., 1990). At present, however, our findings do not directly suggest the existence of such co-activators. First, the data can be simply explained with the existing factors (GAGA factor and repressor) without the inclusion of an additional hypothetical activity. Second, there is a perfect correlation with the action of the repressor and GAGA activation (either in the template commitment assay in Fig. 5 or in the comparison of the HP extract with the 0-50% ammonium sulfate fraction in Fig. 6). Third, the putative auxiliary factor must also commit to the DNA template to accommodate the template commitment data in Fig. 5.

**Role of the DNA Binding Transcriptional Repressor in Vitro and in Vivo**—The importance of the transcriptional repressor has yet to be clearly demonstrated *in vivo*. To address this question, we have compared GAGA factor-mediated transcription activation both *in vivo* and *in vitro* as well as studied the generality of transcriptional antirepression as a mechanism by which sequence-specific transcription factors function. First, we examined GAGA factor-mediated activation of the *Drosophila engrailed (en)* promoter, which contains several GAGA binding sites upstream of the RNA start sites. In transient transfection experiments with the *en* promoter, the amount of transcriptional activation that was contributed by GAGA factor binding sites was roughly 12-fold (see Fig. 6). In contrast, GAGA factor activation of the *en* promoter using identical plasmid constructions was not observed *in vitro* with a GAGA-depleted SK extract (this study, data not shown), which does not contain the transcriptional repressor. GAGA factor-mediated stimulation of transcription was observed, however, with the HP extract, which contains the transcriptional repressor (data not shown). With the SK extract, the lack of transcriptional activation by the GAGA factor indicates that an important component of the normal functioning of the factor is absent, and we suggest, based on the experiment with the HP extract, that the missing component is the transcriptional repressor. We have also begun to examine the generality of transcriptional antirepression (Fig. 6), and it appears that factors other than the GAGA factor, such as GAL4 derivatives and Sp1, function as antirepressors. The generality of antirepression suggests that we have not observed a unique, artificial interaction between the repressor and GAGA factor. We have recently purified the transcriptional repressor to greater than 95% homogeneity, and we hope to use the purified factor to generate reagents, such as antibodies and clones, to examine the role of the repressor in vivo.

Curiously, the general transcriptional repressor that we describe in this work may be related to an inhibitory activity described by DePamphilis and co-workers (Chalifour et al., 1987; Martinez-Salas et al., 1988, 1989). In these studies, they examined promoter and enhancer activity in the one-cell mouse embryo, which contains pronuclei, and the diploid two-cell mouse embryo. In the one-cell embryo, proximal promoters are active in the absence of enhancers, but in two-cell embryos, enhancers are required in addition to the proximal promoter for activity. Hence, in these experiments, it appears that enhancers are functioning to counteract repression by a factor that first arises in the two-cell mouse embryo. This negative factor, which represses transcription from proximal promoters, may be related to the DNA binding repressor that we have characterized in our studies on the *Kr* promoter.

Why hasn't the DNA binding repressor been previously identified? First, transcriptional repression is more difficult to study than activation since there are many nonspecific factors, and "co-activators" are necessary for transcriptional activation by Sp1 (Hoey et al., 1990; Pugh and Tjian, 1990; Peterson et al., 1990) and GAL4-VP16 (Berger et al., 1990; Kelleher et al., 1990). At present, however, our findings do not directly suggest the existence of such co-activators. First, the data can be simply explained with the existing factors (GAGA factor and repressor) without the inclusion of an additional hypothetical activity. Second, there is a perfect correlation with the action of the repressor and GAGA activation (either in the template commitment assay in Fig. 5 or in the comparison of the HP extract with the 0-50% ammonium sulfate fraction in Fig. 6). Third, the putative auxiliary factor must also commit to the DNA template to accommodate the template commitment data in Fig. 5.

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3 W. Soeller and T. Kornberg, manuscript in preparation.

4 W. Soeller, unpublished data.

5 G. E. Croston, L. A. Kerrigan, and J. T. Kadonaga, unpublished data.
means by which levels of RNA can be decreased, such as the action of nucleases, proteases, and phosphatases. Moreover, most procedures for the preparation of extracts for in vitro transcription have been designed to remove factors that inhibit transcription. We have found here that 0-50% ammonium sulfate precipitation of transcription factors, which is commonly used in the preparation of transcription factors, results in loss of the DNA binding repressor. In fact, in vitro extracts are typically indiscriminate with regard to their specificity of transcription. In these instances, it is possible that the absence of an inhibitor, such as the activity that we describe in this work, may be an important factor in the generally promiscuous nature of transcription in vitro.

Does the GAGA factor completely derepress transcription? If the function of the GAGA factor is to prevent transcriptional repression, it may perform this function to varying degrees on different promoters. To investigate this possibility, we have carried out a comparative study in which we examined the relative effectiveness of the GAGA factor and pUC plasmid DNA to counteract transcriptional repression by the inhibitory DNA binding factor. In these experiments, we added variable amounts of either GAGA factor or pUC DNA to transcription reactions (using the GAGA factor-depleted HP extract) and determined the maximal degree of antisense by GAGA factor and pUC. With the Ub promoter, the GAGA factor and pUC stimulated transcription to the same degree, but with Kr, pUC stimulated transcription about twice as effectively as the GAGA factor (data not shown; also see Fig. 5). Hence, it appears that the GAGA factor is more effective at antirepression of Ubx than Kr, and this effect may perhaps be due to the arrangement of GAGA binding sites in the two promoters. The GAGA factor does not appear to derepress the Kr promoter completely, but there may be other factors that also function on the Kr gene in a related manner in vivo.

Relationship between the Nonspecific DNA Binding Repressor and Nucleosome Assembly—What is the relationship between the DNA binding repressor activity in the HP extract and nucleosome assembly? For instance, Workman, Roeder, and co-workers (Workman and Roeder, 1987; Workman et al., 1988) have shown that nucleosomes assembled from partially purified components inhibit transcription in a manner that can be relieved by preincubation with TFIID (the TATA box binding factor) or by preincubation with TFIID and pseudorabies virus immediate early protein. We have addressed the question of nucleosome assembly in our experiments as follows. First, we examined if nucleosome assembly occurs under transcription conditions with the HP extract by measuring supercoiling of relaxed plasmid DNA in the presence of topoisoerase I (Germond et al., 1975). We observed no detectable DNA supercoiling under conditions where purified histones alone with nucleosome assembly factors gave quantitative supercoiling of the plasmid DNA (data not shown). Second, we have found that the GAGA factor does not appear to affect the level of transcription from template DNA assembled with nucleosomes. For these reasons, we do not believe that nucleosome assembly is involved in the transcriptional repression that is relieved by the GAGA factor.

The GAGA Factor—The GAGA factor was originally identified by Biggin and Tjian (1988) in studies on the Drosophila Ultrabithorax promoter. It is a relatively abundant sequence-specific DNA binding protein and has been found to bind to the promoter regions of a variety of Drosophila genes. The GAGA factor interacts with upstream promoter sequences of the Drosophila E74 gene (Thummel, 1989), the Drosophila him3, his4, hsp26, and hsp70 genes (Gilmour et al., 1989), and the Drosophila engrailed gene, but transcriptional stimulation by the GAGA factor has been demonstrated only for Kr and Ubx. Except for Kr, these genes contain GAGA factor binding sites located upstream, but not downstream, of the transcription start sites. We have shown here that the GAGA factor is capable of activating Kr transcription from the downstream binding sites. Transcriptional activation by a proximal promoter DNA binding factor located downstream of the RNA start sites is unusual. Notwithstanding, any proposed model for the mechanism of action of proximal promoter transcription factors should accommodate activation of transcription from downstream of the RNA start sites.

Role of the GAGA Factor in the Regulation of the Kruppel Gene—These studies provide a starting point for the analysis of the transcriptional regulation of the Kruppel gene. In this work, we have focused upon the promoter region in a 1.3-kb SalI to XbaI fragment of the Kr gene that encompasses positions -861 to +426 relative to the major upstream start site. We have found that the GAGA factor binds to multiple sites in this region and stimulates transcription of the Kr gene in vitro. Thus, the GAGA factor is probably a constituent of the active promoter in vivo, but is it also involved in the transcriptional regulation of the Kr gene? Studies on the mechanism of action of the GAGA factor in Drosophila embryos expressing a polyubiquitinated antibody against the GAGA factor have revealed that it is uniformly distributed in every nucleus in the embryo. Hence, it is unlikely that the GAGA factor is involved in the spatial regulation of Kr transcription in the blastoderm. However, GAGA factor expression is highly regulated over different stages of Drosophila development, and, thus, the GAGA factor may have a role in the temporal regulation of Kr expression.

The cis-acting transcriptional control elements of the Kr gene have been analyzed by P element-mediated germ line transformation experiments (Hoch et al., 1990). In this work, Hoch et al. have found that the 1.3-kb SalI-XbaI Kr fragment, which was used in our studies, contains promoter activity and specifies transcription in the nervous system. Moreover, they have also identified an enhancer element located several kilobases upstream of the transcription start sites that is important for spatial regulation of Kr transcription in the blastoderm. These findings have enabled us to initiate an analysis of the trans-acting factors that interact with the upstream enhancer. We hope, in the future, to link our biochemical studies on the Kr promoter and enhancer regions with the P element transformation data to determine the role of individual factors in the expression of the Kr gene. In this manner, we hope that we may gain a better understanding of an important step in the development of the embryo.

Acknowledgments—We are most grateful to Michael Hoch and Herbert Jackie for the Kruppel gene and communication of unpublished information; Mark Biggin for the Ubx promoter mutants and helpful suggestions in the course of this work; Walter Soeller and Tom Kornberg for pα-906DM26, pα-380DM26, and communication of unpublished information; Mike Carey for plasmids encoding GAL4-VP16 derivatives and GAL4-responsive promoters; Mel DePamphils for suggesting the possible relationship between the non-specific transcriptional repressor and the negative regulatory factor that appears in two-cell mouse embryos; Paul Laybourn for assistance and advice on nucleosome assembly and histones; Robinton Kamakaka for the gift of GAL4-VP16 protein; and Kathy Jones, Debbie Spector, and members of the Kadonaga lab for critical reading of the manuscript. We also thank Donna Bautista and Brian Martin for excellent technical assistance.

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