Riggs, A. (1981) *Methods Enzymol. 76*, 8.
Ringe, D., Petsko, G. A., Kerr, D. E., & Ortiz de Montellano, P. R. (1984) *Biochemistry 23*, 2.
Sampath, V., & Caughey, W. S. (1985) *J. Am. Chem. Soc. 107*, 4076.
Satterlee, J. D. (1983) *Inorg. Chim. Acta 79*, 170.
Satterlee, J. D., Teintze, M., & Richards, J. H. (1978) *Biochemistry 17*, 1456.
Sharma, V. S., & Ranney, H. M. (1982) *J. Mol. Biol. 158*, 551.
Sharma, V. S., Vedvick, T. S., Magde, D., Luth, R., Friedman, D., Schmidt, M. R., & Ranney, H. M. (1980) *J. Biol. Chem. 255*, 5879.
Shimada, H., & Caughey, W. S. (1982) *J. Biol. Chem. 257*, 11893.
Shimada, H., Dong, A., Ishimura, Y., & Caughey, W. S. (1989) *Biochem. Biophys. Res. Commun. 158*, 110.
Smith, M. L., Paul, J., Ohlson, P. I., & Paul, K. G. (1984) *Biochemistry 23*, 6776.
Stenzel, P., Brimhall, B., Jones, R. T., Black, J. A., McLachian, A., & Gibson, D. (1979) *J. Biol. Chem. 254*, 2071.
Tucker, P. W., Phillips, S. E. V., Perutz, M. F., Houtchens, R., & Caughey, W. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1076.
Wallace, W. J., & Caughey, W. S. (1975) *J. Am. Chem. Soc. 107*, 4076.
Yoshikawa, S., O'Keeffe, D. H., & Caughey, W. S. (1985) *J. Biol. Chem. 260*, 3518.
Yu, N.-T., Kerr, E. A., Ward, B., & Chang, C. K. (1983) *Biochemistry 22*, 4534.

High Mobility Group Proteins 1 and 2 Function as General Class II Transcription Factors†

Jagmohan Singh*,‡ and Gordon H. Dixon

Department of Medical Biochemistry, Health Sciences Center, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta T2N 4N1, Canada

Received February 7, 1990; Revised Manuscript Received March 27, 1990

ABSTRACT: High mobility group (HMG) proteins 1 and 2 are thought to be associated with chromatin enriched in active gene sequences, to stimulate endogenous transcription of class II and III genes using HMG-depleted nuclei, and to bind specific DNA sequences upstream of the coding regions of trout HMG-T and human β-globin genes. In testing the possibility that these proteins may act as general transcription factors, the run-off transcription of trout protamine, human β-globin, adenovirus 2 major late promoter, and herpes simplex virus (HSV) thymidine kinase genes was found to be inhibited by affinity-purified HMG-1 and -2 antibodies. The inhibition was partially relieved by exogenously added HMG-1 or -2. A complementation assay showed that the 0.15 M KCl flowthrough of HeLa nuclear extract fractionated by anion-exchange chromatography (DE-52) could be replaced by purified HMG-1 and/or -2 to complement transcription of the trout protamine gene by the 0.5 M KCl eluate fraction. Inhibition studies with heparin showed that HMG-1 and -2 were required for initiation of transcription. These results indicate an absolute requirement of HMG-1 and -2 for class II gene transcription. Western blotting and transcription reconstituted with purified factors show a copurification of HMG-1 and -2 with factor II B, described earlier by Reinberg and Roeder [(1987) *J. Biol. Chem. 262*, 3310–3321].

The structure, localization, and function of high mobility group proteins have been extensively studied since their discovery by E. W. Johns [reviewed in Johns (1982)]. Their presence in a variety of eukaryotic species (Mayes, 1982) suggests that they perform some important biological function(s). Moreover, the genetic inactivation of the gene for a yeast HMG-like protein (ACP-2) with sequence similarity to HMG-1/-2 has recently been shown to be lethal (Haggren & Kolodrubetz, 1988). Dixon and co-workers (Hutcheon et al., 1980; Levy et al., 1977a,b) and Weintraub and co-workers (Weintraub & Groudine, 1976; Weisbrod & Weintraub, 1980) showed that the smaller HMG's, namely, HMG-14 and -17 (molecular weight ≈10 000 each) and H6, their trout counterpart, were preferentially associated with transcriptionally active genes. Weintraub and co-workers showed that at the level of both intact chromatin and isolated nucleosomes of chicken reticulocytes, the DNase 1 sensitivity of the active globin gene was due to association with HMG-14 and -17 (Mardian et al., 1980; Weisbrod et al., 1980). How HMG-14 and -17, which appear to bind to DNA at the entry and exit to the nucleosome (Mardian et al., 1980), could recognize potentially active nucleosomes as opposed to the bulk, inactive nucleosomes was not clear. Recently, antibody probing has shown that HMG-17 is localized on chromatin of transcribed genes only downstream from the starting point of transcription (Dorbic & Wittig, 1987).

In contrast with the studies on HMG-14 and -17 and H6, no clear function has been established for the larger HMG's, HMG-1 and -2 (molecular weight ~25 000–30 000). They have been shown to be associated with nucleosomes of active genes (Jackson et al., 1979; Vidali et al., 1977), and HMG-T, their trout counterpart, appeared, by antibody binding, to be

† This work was supported by grants from the Alberta Heritage Foundation for Medical Research and the Medical Research Council of Canada.
* Author to whom correspondence should be addressed.
‡ Present address: NCI–Frederick Cancer Research Facility, BRI–Basic Research Program, P.O. Box B, Frederick, MD 21701.

0006-2960/90/0429-6295$02.50/0

associated with internucleosomal linker regions (Levy et al., 1977a,c). The relative abundance (10^5–10^6 molecules/nucleus) of HMG-1 and -2 suggests a general structural rather than a gene-specific role (Levy et al., 1977c; Duguet & DeRecondo, 1978). An interesting property of HMG-1 and -2, by which they are able to induce negative supercoiling in relaxed plasmids (Javaherian et al., 1978a,b), suggests that these proteins could play a role in replication, transcription, or recombination. This is in agreement with the observed elevation of HMG-2 levels in replicating and meiotic tissues (Bucci et al., 1984; Seyedin & Kistler, 1979) and stimulation of activities of homologous α- and β-type DNA polymerases by the HMG proteins from regenerating rat liver (Duguet et al., 1977). A role in transcription is suggested by reduction of endogenous RNA synthesis by RNA polymerases II and III upon depletion of HMG's from myeloma nuclei and its restoration by addition of purified HMG-1 and -2 (Stoute & Marzluff, 1977). Similarly, injection of calf thymus HMG-1 antibodies into amphibian oocytes resulted in inhibition of nonnucleolar RNA synthesis and retraction of the lateral loops of the lampbrush chromosomes (Kleinschmidt et al., 1983). Purified HMG-1 and -2 have also bee shown to cause a nonspecific stimulation of transcription by RNA polymerases II and III in vitro (Tremethick & Molloy, 1986).

Recently Wright and Dixon (1988) showed that the trout HMG-T and calf thymus HMG-1 can bind to specific AT-rich sequences upstream of the trout HMG-T and human β-globin genes. Using the in vitro transcription system of Dignam et al. (1983), we show here that the affinity-purified antibodies to HMG-1 and -2 inhibit specific transcription of several class II genes, suggesting that HMG proteins 1 and 2 can function as general positive transcription factors for class II genes. Moreover, HMG antibody reacts specifically with enriched factor IIB, and its inhibition of transcription is relieved by factor IIB, suggesting a possible identity of HMG-1 and -2 with proteins present in factor IIB preparations (Reinberg & Roeder, 1987).

Materials and Methods

Purification of HMG Proteins 1 and 2. HMG-1 was purified from calf thymus chromatin by PCA extraction and CM-Sephadex C-25 chromatography according to Nicolas and Goodwin (1982) or by 0.35 M NaCl extraction of calf thymus chromatin and phosphocellulose chromatography (Isackson et al., 1980). The pooled fractions were dialzyed against 0.1 mM PMSF in water at 4 °C, lyophilized, dissolved in 10 mM HEPES (pH 7.9)/10 mM KCl/0.2 mM DTT/0.25 mM PMSF/10% glycerol, and stored at −20 °C. The phosphocellulose-purified HMG's were much more active than those purified by PCA extraction. However, the HMG's purified by phosphocellulose chromatography were susceptible to proteolysis. Therefore, except for the initial experiments, the PCA extraction method was employed.

Antibodies and Immunoblotting. Five to ten micrograms of purified HMG-1 and -2 was mixed with complete Freund's adjuvant and injected subcutaneously into rabbits at weekly intervals for 3 weeks, followed by a booster with HMG mixed with incomplete adjuvant. The sera obtained after bleeding 1 week later were precipitated with ammonium sulfate at 50% saturation. The precipitate was washed with ammonium sulfate several times and dissolved in Tris–saline, dialyzed against water at 4 °C, and lyophilized.

Purified calf thymus HMG-1 was covalently linked to cyanogen bromide activated Sepharose (Pharmacia) according to manufacturer's directions. Affinity purification of HMG-1/-2 antibodies was done according to Bustin and Neihart (1979). For immunoblotting, the protein samples were resolved by electrophoresis on a 15% polyacrylamide–SDS slab gel (Laemmli, 1970) and transferred by electroblotting to nitrocellulose (Schleicher & Schuell) in the presence of 20 mM Tris, 150 mM glycine, pH 8.4, and 20% methanol at 10 V for 6–8 h or overnight. The blotted proteins were detected by the alkaline phosphatase conjugated anti-rabbit IgG detection system (Promega) according to manufacturer's directions.

Preparation of HeLa Nuclear Extracts. Four liters of HeLa cells was grown in suspension up to a density of (4–5) × 10^5 cells/mL. The cells were harvested and the nuclear extracts prepared and stored according to Dignam et al. (1983).

Specific Transcription Reactions. The conditions for specific transcription, complex formation, and electrophoretic analysis of the transcription products were as described by Reinberg and Roeder (1987). Conditions for transcription reconstitution using the $pML(C_2AT)_{19}$ template and purified factors have been described (Flores et al., 1988; Sawadogo & Roeder, 1985).

DNA Templates. The human β-globin clone pβBam contains a 1.94-kb insert cloned in pBR322. After *Bam*HI digestion, these templates would be expected to yield a 478-nucleotide (nt) run-off transcript. The trout protamine gene pJP22 (Jankowski & Dixon, 1984) when linearized with *Bam*HI gives a 503-nt transcript. Plasmid pSmaF contains the SmaF fragment of the adenovirus major late promoter (Ad2 MLP) cloned into the unique *Nru*I site of pBR322. It was digested with *Ava*II, and the expected run-off transcript is 805 nt. Plasmid pMWTK contains the thymidine kinase gene of herpes simplex virus type 1. It was digested with *Hin*fI, the expected run-off transcript size being 807 nucleotides. The construction of the $MLP(C_2AT)_{19}$ template has been described by Sawadogo and Roeder (1985).

Extraction of HMG Proteins from HeLa Nuclear Extracts. The nuclear extracts were precipitated with 20% TCA or subjected to fractional 3–20% TCA precipitation. The pellets were washed with chilled acid–acetone (100 μL of concentrated HCl/200 mL of acetone) and acetone and air-dried. The dried protein was dissolved in acid–urea sample buffer and resolved by acetic acid–urea–polyacrylamide (15%) gel electrophoresis according to Panyim and Chalkey (1969).

DE-52 Chromatography. The HeLa nuclear extract (1 mL) was adjusted to 0.15 M KCl by adding 1.0 M KCl and passed down a 0.2-mL column of DEAE-cellulose (DE-52, Whatman). After the column was washed with 0.2 mL of buffer D/0.15 M KCl (Dignam et al., 1983) and the flowthrough (FT1) was collected, the bound protein was eluted with 1 mL of 0.5 M KCl in buffer D (Dignam et al., 1983). The 0.5 M KCl eluate was diluted 4-fold with buffer D containing no KCl (EL1). Then 0.2 mL of 0.15 M KCl flowthrough fraction was passed through another 0.4-mL DE-52 column equilibrated against buffer D/0.15 M KCl. The flowthrough was collected and pooled with the 0.4-mL wash of the column with buffer D/0.15 M KCl to give the FT2 fraction (dilution factor with respect to the extract being 3.6). The proteins bound to DE-52 were eluted with 2.4 mL of buffer D/0.5 M KCl to give the EL2 fraction (the dilution factor being 12). All fractions, i.e., FT1, EL1, FT2, and EL2, were frozen in aliquots in liquid nitrogen and stored at −70 °C.

Results and Discussion

Purity of HMG-1 and -2 and Antibody Specificity. In preliminary experiments, the ability of HMG antibody to inhibit specific transcription in vitro and whether this inhibition could be relieved by exogenously added HMG-1 and -2 were examined. For this experiment, it is important to prepare

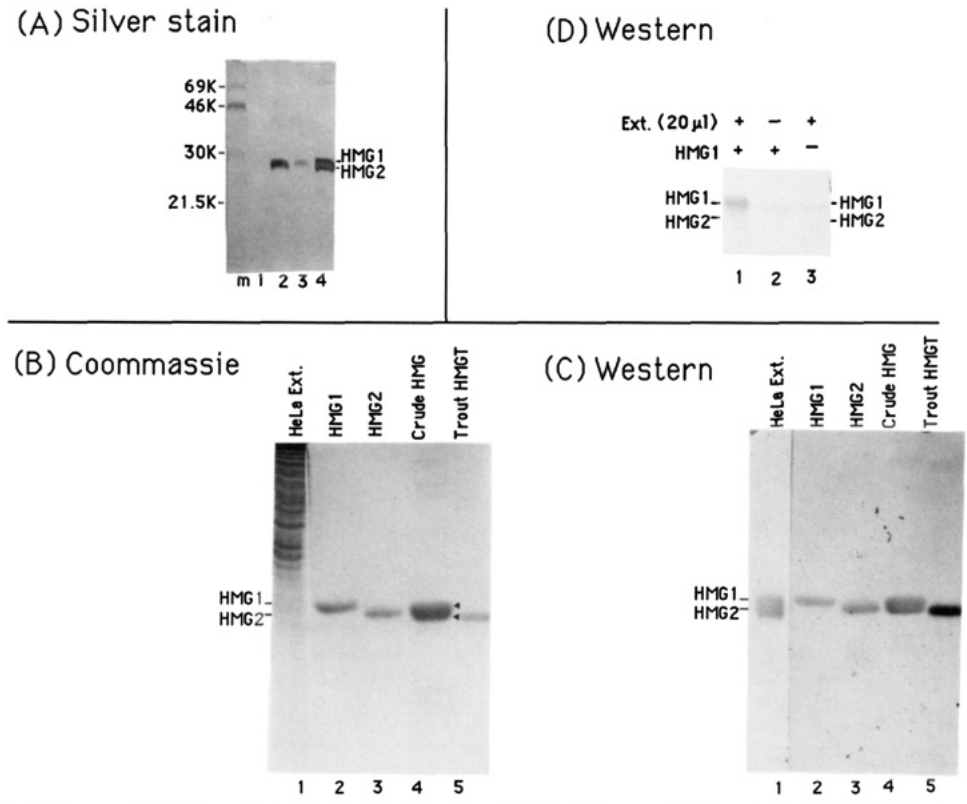

FIGURE 1: Purity of calf thymus HMG-1 and -2 and specificity of the HMG affinity-purified antibody. (A) HMG-1 and -2 were resolved at two different amounts on a 10% SDS–polyacrylamide gel and visualized by silver staining. m, 0.5 µg of the BRL rainbow molecular weight markers; lanes 1 and 2 have 0.15 and 1.35 µg of HMG-1, respectively, and lanes 3 and 4 have 0.30 and 2.7 µg of HMG-2, respectively. (B and C) Western blot analysis of HeLa extract and HMG proteins, (B) represents the Coomassie-stained gel and (C) the Western blot: lane 1, 10 µL of HeLa extract; lane 2, 1.25 µg of calf thymus HMG-1; lane 3, 1.25 µg of calf thymus HMG-2; lane 4, crude preparation of calf thymus HMG-1 and -2; lane 5, 1 µg of trout HMG-T. After proteins were resolved on a 15% SDS–polyacrylamide gel and transferred to a nitrocellulose filter, the HMG affinity-purified antibody was used at 0.5 µg/mL, for 1 h at room temperature, followed by treatment with alkaline phosphatase conjugated goat anti-rabbit antibody (Promega) for 30 min and color development according to manufacturer's directions. (D) Western blot analysis of the HeLa extract (20 µL, lane 3), HMG-1 (200 ng, lane 2), and 20 µL of extract plus 500 ng of HMG-1 (lane 1). Proteins were resolved on a 10% SDS–polyacrylamide gel and processed for Western blotting as described under Materials and Methods.

highly purified HMG-1 and -2 proteins and a highly specific antibody. The homogeneity of HMG-1 and -2 purified by phosphocellulose chromatography was checked by SDS–polyacrylamide gel electrophoresis followed by silver staining. As shown in Figure 1A, both HMG-1 and -2 appear homogeneous. The additional high molecular weight band seen in lane 4 (Figure 1A) at higher loading of HMG-2 corresponds to a multimer of HMG-2, which we have often observed in preparations of HMG-1, -2, or HMG-T that are purified under nonreducing conditions, and is fully converted to the faster migrating band on incubating in the presence of β-mercaptoethanol. The specificity of the affinity-purified antibody against HMG-1 and -2 was checked by Western blotting. As shown in Figure 1B (Coomassie staining) and Figure 1C (immunoblot), the antibody reacts with calf thymus HMG-1 and -2 (lanes 2 and 3, Figure 1B,C), which share considerable sequence homology (37), as well as with the trout HMG-T (lane 5, Figure 1B,C). The antibody also reacts with two proteins in the HeLa extract (lane 1, Figure 1B,C), which comigrate with calf thymus HMG-1 and -2 (compare lane 1 with lanes 2 and 3 in Figure 1B,C). Moreover, when calf thymus HMG-1 was added to the HeLa extract in a Western blot shown in Figure 1D, the intensity of the upper band present in the HeLa extract was increased (compare lanes 1 and 3 in Figure 1D), suggesting that the two protein bands observed in the HeLa extract correspond to HeLa HMG-1 and -2. Thus, the antibody displays a monospecificity toward HMG-1 and -2, and there is no contaminating antibody of different specificity. Therefore, both the antibody and the purified preparations of HMG-1 and -2 are useful reagents to test the requirement of HMG-1 and -2 for transcription.

HMG Antibody Inhibits the in Vitro Run-Off Transcription of Class II Genes by HeLa Nuclear Extracts. The effect of preincubation of the HMG affinity-purified antibody with the HeLa nuclear extract on the run-off transcription of human β-globin gene was tested in a run-off transcription assay according to Reinberg and Roeder (1987). A 478-nucleotide (nt)-long run-off transcript was synthesized by the control extract (lane 1, Figure 2). Preincubation of the extract with 1 µg of specific HMG antibody completely inhibited the transcription of the specific 478-nt RNA (lane 2, Figure 2), while 15 µg of preimmune IgG had no effect (lane 3, Figure 2). When the HMG antibody was first preincubated with 0.5 and 1 µg of HMG-1 (that was freshly purified by phosphocellulose P-11 chromatography) before addition to the HeLa extract (see the scheme at the top left of Figure 2), there was a progressive and complete restoration of the levels of the specific transcript (lanes 4 and 5, Figure 2), as assayed by densitometric scanning of the autoradiogram and expressed in absorbance units below the lanes in Figure 2. The synthesis of the 478-nt transcript was sensitive to specific polymerase II inhibitors, 5 µg/mL heparin (lanes 7 and 8, Figure 2) as well as 1 µg/mL of α-amanitin (not shown). When heparin was added at the same time as the DNA template, there was complete inhibition of specific transcription (lane 8, Figure 2), but if heparin was added 1 min after the template DNA, a small but detectable level (2–5% of control) of specific transcript was observed (lane 7, Figure 2), which results from

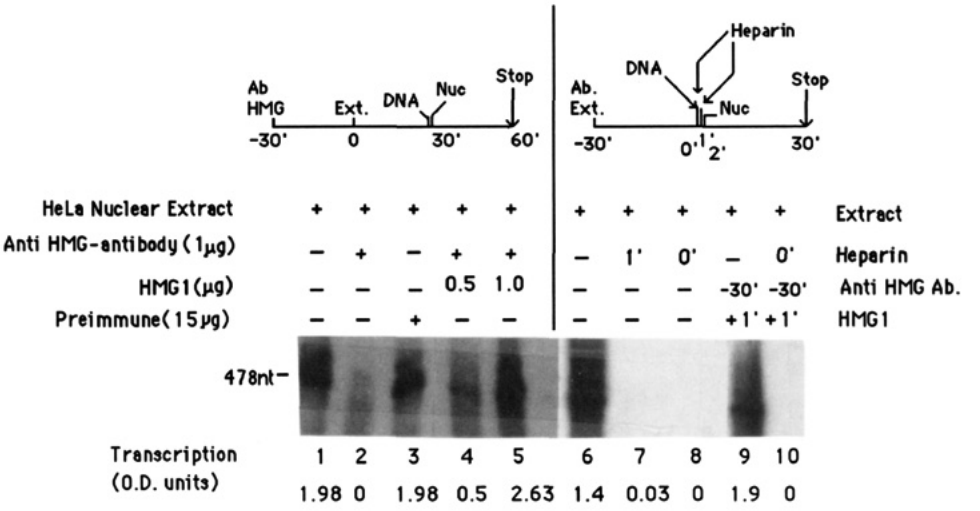

FIGURE 2: Inhibition of specific run-off transcription of human β-globin gene by HMG affinity antibody. Lane 6, 3 μL of extract was incubated with 0.5 μg of pBBam DNA digested with *Bam*HI, followed by addition of 600 μM each of ATP, CTP, and GTP and 5 μCi of [α-^{32}P]UTP (650 μCi/mmol, ICN) at 30 °C for 30 min in a 30-μL reaction volume, followed by addition of 80 μL of stop mix, phenol–chloroform extraction, and ethanol precipitation. The transcription products were resolved by denaturing urea–acrylamide gel electrophoresis, after which the gels were dried and autoradiographed. In lane 2, the extract was preincubated with 1 μg of HMG antibody for 30 min at 30 °C before addition of template and nucleotides. In lane 3, 15 μg of preimmune IgG was used. In lanes 4 and 5, 0.5 and 1 μg of HMG-1 were preincubated with affinity antibody for 30 min at 30 °C and then added to 3 μL of the HeLa extract and incubated for 30 min further at 30 °C, and then the template and nucleotides were added for the transcription incubation. In lane 7, the template was added to 3 μL of extract at zero minutes, while heparikn was added at 1 min or zero minutes (lane 8), and at 1 min, the nucleotides were added, and incubation was carried out at 30 °C for 30 min. In lanes 9 and 10, the extract was incubated with HMG antibody for 30 min and the template added at 0 min, HMG-1 at +1 min, and nucleotides at 2 min and incubation done at 30 °C for 30 min. In lane 10, heparin was added at 0 min. After autoradiography at −70 °C, the lanes were scanned, and the levels of transcription were quantitated with a densitometer and expressed as OD units. Lane 1, ^{32}P end-labeled BRL RNA size markers.

the elongation of the heparin-resistant transcription initiation complex formed in the 1 min prior to addition of heparin, as previously shown by Reinberg and Roeder (1987). Moreover, if HMG-1 was added to the extract that had been preincubated with the HMG antibody 1 min after the DNA template (lane 9, Figure 2) but not if added 1 min after heparin addition (lane 10, Figure 2), a complete restoration of transcription to the control level was observed, suggesting that HMG-1 (and/or -2) is required for the formation of the heparin-resistant transcription initiation complex.

To test if HMG-1 and/or -2 are required for transcription of other class II genes, the effect of HMG antibody and run-off transcription of the trout protamine gene pJP22, Ad2MLP-pSmaF, and HSV thymidine kinase genes was tested. As shown in Figure 3, HMG antibody inhibited the transcription of pJP22 (lanes 2 and 3, Figure 3A), pSmaF (lane 3, Figure 3B), and Tk (lane 3, Figure 3C) genes. The transcription of pJP22 (lane 7, Figure 3A) as well as pSmaF and Tk (not shown) was sensitive to 1 μg/mL α-amanitin. High concentrations of preimmune IgG (15 μL/reaction) had no effect on specific transcription of any of these (lane 6, Figure 3A, lane 2 in Figure 3B,C). Upon preincubation of antibody with exogenous HMG-1 (which was purified by the PCA extraction method), followed by renaturation before incubation with the nuclear extract, a partial restoration of transcription of all three genes was observed (lane 5, Figure 3A; lane 4, Figure 3B; and lanes 4–6, Figure 3C).

There is extensive premature termination of transcription observed in the cases of pSmaF and Tk genes. However, this is a usual occurrence in run-off transcription experiments (Reinberg & Roeder, 1987); for example, multiple shorter transcripts were also observed in addition to the correct size transcript during transcription of the Tk gene by Jones et al. (1985) and McKnight (1982). Such premature termination might also result from the longer length of the transcription unit. Densitometric quantitation of the autoradiograms shows restoration of ~24% and 12%, respectively, of the transcription of pJP22 and Ad2MLP genes, and for TK genes, it was estimated to be 10.8%, 30.6%, and 6.1% for 0.24, 0.48, and 0.72 μg, respectively, of calf thymus HMG-1 used in relieving antibody inhibition of transcription. A similar extent of restoration by RAP30 from inhibition of transcription with HeLa extract by anti-RAP30 antibody has been reported by Burton et al. (1988). The reason for the limited restoration of transcriptional activity by the addition of purified HMG's both in our experiments and in those of Burton et al. (1988) is not yet clear. One possibility was that, if HMG forms part of the initiation complex then the HMG antibody may coprecipitate other transcription factor proteins which would not be replaced when pure HMG's were added. However, results of immunoprecipitation of ^{35}S-labeled HeLa nuclear extract by anti-HMG antibody failed to show precipitation of any protein other than HMG-1 and -2 (not shown). Thus, the possibility of coprecipitation can be discounted. The most likely explanation for the partial restoration is a partial loss of activity in the harsh purification procedure. Besides, transcription factors purified to homogeneity are known to undergo a progressive loss of activity (Sawadogo, 1988). Moreover, the fact that HMG-1 that was freshly purified by the nondenaturing phosphocellulose method could fully relieve the antibody inhibition of run-off transcription of human β-globin gene (Figure 2) also argues against the possibility of coprecipitation. Owing to the inability to consistently obtain undegraded HMG's by the phosphocellulose method because of degradation during salt extraction of calf thymus chromatin (Nicolas & Goodwin, 1982), for most of the experiments, HMG-1 and -2 were purified to homogeneity by PCA extraction/CM-Sephadex C-25 chromatography (Johns, 1982), followed by incubation with buffer containing DTT at room temperature to renature the protein.

An intriguing result shown in Figure 3C is inhibition of transcription at higher HMG concentrations (lanes 5 and 6).

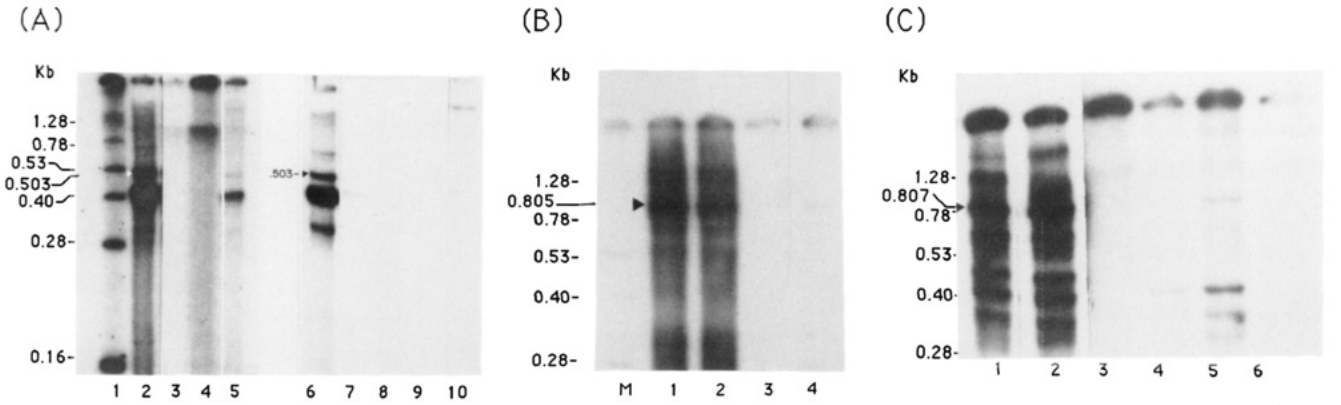

FIGURE 3: HMG-1 and -2 affinity antibody inhibits the accurate run-off transcription of several class II genes. (A) pJP22–*Bam*HI (0.5 μg). (B) Ad2MLP–pSmaF–*Ava*II digest (0.5 μg) and (C) thymidine kinase gene–*Hin*fI digest (0.5 μg) DNAs were used as templates with 3 μL of HeLa nuclear extract in a 30-μL reaction volume. (A) Lane 2, control extract alone was incubated with pJP22–*Bam*HI digest to give a 503∞nt transcript; lanes 3, 4, and 6, 1 and 2 μg of HMG antibody and 15 μg of preimmune IgG were preincubated with HeLa extract for 30 min at 30 °C, followed by transcription reaction; lane 5, 0.48 μg of HMG-1 purified by the PCA extraction method was preincubated at 30 °C for 30 min in 10 mM HEPES (pH 7.9)/10 mM KCl/0.5 mM DTT/0.25 mM PMSF, then added to 2 μg of HMG antibody in transcription buffer, and incubated at 30 °C for 20 min and then at 4 °C for 10 min before addition of 3 μL of extract and further incubation at 4 °C for 60 min. The samples were then brought to room temperature, template DNA and nucleotides were added, and incubation for transcription was carried out. (Lane 7) Sarkosyl at 2.5%, (lane 8) ammonium sulfate at 0.2 M, (lane 9) heparin at 5 μg/mL, and (lane 10) α-amanitin at 1 μg/mL were added to the extract before starting the incubation with pJP22 DNA and nucleotides. (B) Ad2MLP-pSmaF DNA digested with *Ava*II was used as template (0.5 μg/reaction). The expected run-off transcript is 805 nt long: lane 1, control extract alone; lane 2, 15 μg of preimmune IgG was preincubated with extract for 30 min at 30 °C; lane 3, 1 μg of HMG antibody was incubated with HeLa extract in transcription buffer at 30 °C for 30 min; lane 4, 0.48 μg of HMG-1 was preincubated with 1 μg of HMG antibody for 30 min at 30 °C and then 3 μL of extract added and incubation done as in (A). Finally, the template DNA was added and the transcription reaction carried out. (C) HSV-TK DNA (0.5 μg) digested with *Hin*fI was used as template, to give a 807-nt-long run-off transcript. Lane 1, control extract (3 μL) alone; lane 2, 15 μg of preimmune IgG was preincubated with extract in transcription buffer at 4 °C for 60 min; lane 3, 1 μg of HMG antibody was incubated with extract in transcription buffer at 4 °C for 60 min. In lanes 4, 5, and 6, 1 μg of HMG antibody was preincubated with 0.24, 0.48, and 0.72 μg, respectively, of renatured calf thymus HMG-1 (purified by the PCA extraction method) for 30 min at 30 °C and 3 μL of extract added and incubation continued at 4 °C for 60 min. After the incubation, the template DNA and nucleotides were added, and the transcription reaction was carried out.

This effect may be explained by the "Squelching" phenomenon, originally reported by Gill and Ptashne (1988), whereby an excess of a positive transcription factor, like GAL4, can interact with another DNA binding transcription factor and prevent the latter from binding to its DNA recognition site. In the case of HMG's, such a scenario would suggest that HMG-1 and -2 can specifically interact with one more of the known class II transcription factors.

To further rule out the possibility of coprecipitation, an alternative complementation assay was devised. Using an anion-exchanger column (DE-52), it was found that endogenous HMG-1 and -2 of the HeLa nuclear extract [as characterized by their solubility in 3% TCA and precipitation by 20% TCA, and electrophoretic mobility in acid–urea–polyacrylamide gel, where they comigrate with purified calf thymus HMG-1 and -2 (Figure 4A) (Johns, 1982; Mayes, 1982)] were not bound to the column at 0.15 M KCl in buffer D (lanes 3 and 6, Figure 4A) and appeared in the flowthrough fractions, FT1 and FT2. It has been shown by Dignam et al. (1983) that most of the class II transcription factors (except factors IIB and IIS) present in HeLa extract are bound to the DE-52 column at KCl concentrations greater than 0.10–0.15 M. Therefore, it was thought that if HMG-1 and -2 do act as specific transcription factors, they should be able to replace the 0.15 M KCl flowthrough, FT2 (which contains HeLa HMG-1 and -2; Figure 4A), in complementing transcription by the 0.15–0.5 M KCl eluate fraction, EL2, which contains other proteins essential for transcription that are bound at 0.15 M KCl but eluted by 0.5 M KCl (lane 7, Figure 4A). It was found that the first 0.15 M KCl flowthrough, FT1, was as active as the control extract (not shown), which is understandable since the protein complement of FT1 is both qualitatively and quantitatively similar to that of control extract (compare lanes 3 and 2 in Figure 4A). The first eluate, EL1, had a slight activity. This suggests that most proteins were not bound to the column because of overloading. Therefore, FT1 and EL1 were unsuitable for the complementation assay. However, the second 0.15 M KCl flowthrough (FT2) and 0.5 M KCl eluate (EL2) fractions obtained after passing FT1 through a bigger DE-52 column (see Materials and Methods) were both inactive separately in accurate transcription of pJP22 (lanes 2 and 3, Figure 4B). The protein analysis of these fractions showed that, while FT2 consisted primarily of HMG-1 and -2 (lane 6, Figure 4A), EL2 contained a heterogeneous mixture of proteins (lane 7, Figure 4A). Both FT2 and EL2 fractions were transcriptionally inactive, but the FT2 fraction could restore accurate transcription when added together with the EL2 fraction (arrow, lane 4, Figure 4B). Interestingly, both HMG-1 and -2 (apparently homogeneous preparations purified by Mono Q/Mono S chromatography, a gift of G. R. Reeck; Abdul-Razzak et al., 1987), when added to the EL2 fraction at their estimated levels in the HeLa extract (see figure legend), each could separately restore the transcription to an extent comparable to FT2 (lanes 5 and 6, Figure 4B). The presence of short transcripts (Figure 4B) might result from premature termination, possibly because of the lack of elongation factor IIS, which, according to Dignam et al. (1983), is present in the DE-52 flowthrough fraction, the consequent lack of which in EL2 might cause premature termination. The relatively lower level of transcription in the complementation assay as compared to the control extract is expected since the design of the experiment leads to partial removal of some of the factors in the first eluate fraction (EL1; as is obvious in lane 4 of Figure 4A), which were bound to the first DE-52 column and eluted at 0.5M KCl but not pooled with the EL2 fraction in the transcription complementation experiment. It was also observed that the control transcription was similar in 30- and 70-μL reaction

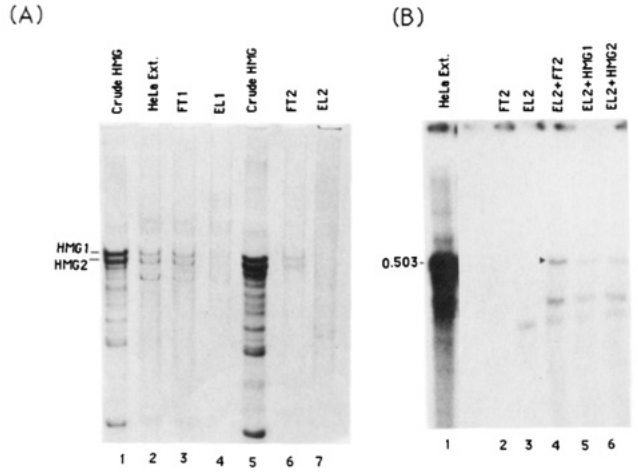

FIGURE 4: DE-52 chromatography and transcription complementation assay. (A) Acetic acid–urea–polyacrylamide gel (15%) analysis of the different DE-52 fractions of the HeLa nuclear extract (see Materials and Methods). Lanes 1 and 5, 50 μg of the 3–20% TCA-precipitable fraction of the salt extract of calf thymus chromatin; lane 2, 40 μL of the control HeLa extract; lane 3, FT1 fraction (48 μL); lane 4, EL1 fraction (200 μL); lane 6, FT2; lane 7, EL2 fraction (1 mL); volumes of FT1, FT2, EL1, and EL2 were increased to allow for dilution. All the DE-52 fractions were precipitated with TCA at 20% concentration and samples prepared for electrophoresis as described under Materials and Methods. (B) Transcription complementation assay using the trout protamine gene (pJP22). The transcription reactions were carried out in a volume of 70 μL. Lane 1, 3.0 μL of control extract; lane 2, 10.8 μL of FT2; lane 3, 36 μL of EL2; lane 4, 10.8 μL of FT2 plus 36 μL of EL2; lane 5, 36 μL of EL2 plus 210 ng of calf thymus HMG-1; lane 7, 36 μL of EL2 plus 250 ng of calf thymus HMG-2 (dilution factor for FT2 ~3.6; for EL2 ~12). The fractions were added to the transcription buffer and incubated at 4 °C for 10 min followed by addition of pJP22 DNA (0.5 μg) and nucleotides and incubation at 30 °C for 30 min.

volumes; the higher KCl concentrations (upto 60 mM) obtained, due to the larger volume of EL2 used, did not affect the level of transcription (not shown). Complementation assay with human β-globin gene gave similar results. These results unequivocally show that HMG-1 and -2 are absolutely required for specific transcription of class II genes.

HMG-1 and -2 Copurification with Transcription Factor IIB. To test if HMG-1 and -2 are identical with any of the known class II transcription factors, the known chromatographic properties of HMG-1 and -2 were compared to those of the previously characterized transcription factors. A striking similarity was observed between HMG-1 and -2 and TF-IIB. HMG-1 and -2 elute from phosphocellulose (Isaackson et al., 1980), ssDNA–agarose (Isaackson et al., 1979), and DE-52 (this paper) at similar ionic strength as factor IIB (Reinberg & Roeder, 1987) and migrate as a doublet of molecular weight of 25 000–28 000 on the SDS–polyacrylamide gel, like factor IIB (Reinberg & Roeder, 1987). To test if HMG-1 and -2 are immunologically and functionally similar to or identical with factor IIB, cross-reaction of the HMG affinity-purified antibody with factor IIB and factor IIB sensitive inhibition of specific in vitro transcription using purified or enriched factors were checked. As shown in Figure 5, the HMG antibody reacts with two protein bands in the HeLa extract, which are also present in the 0.5 M KCl eluate step from phosphocellulose P-11, the IIB fraction of a Superose sizing column, and the 0.25 M KCl eluate (which has the IIB activity) of the ssDNA–agarose column, and comigrate, as previously shown in Figure 1B–D, with calf thymus HMG-1. Reinberg and Roeder (1987) have shown that the ssDNA–agarose column fraction contains only three to four protein bands as visualized by silver staining on an SDS–polyacrylamide gel. Two of these correspond to HMG-1 and -2, as shown by the Western blotting data in Figure 5. No immunologically cross-reacting protein is present in the major late transcriptional factor preparation (MLTF), factor IIE/F (Reinberg & Roeder, 1987), the 0.1 M KCl wash fraction of phosphocellulose P-11 (factor IIA), factor IID, and RNA polymerase II (Figure 5).

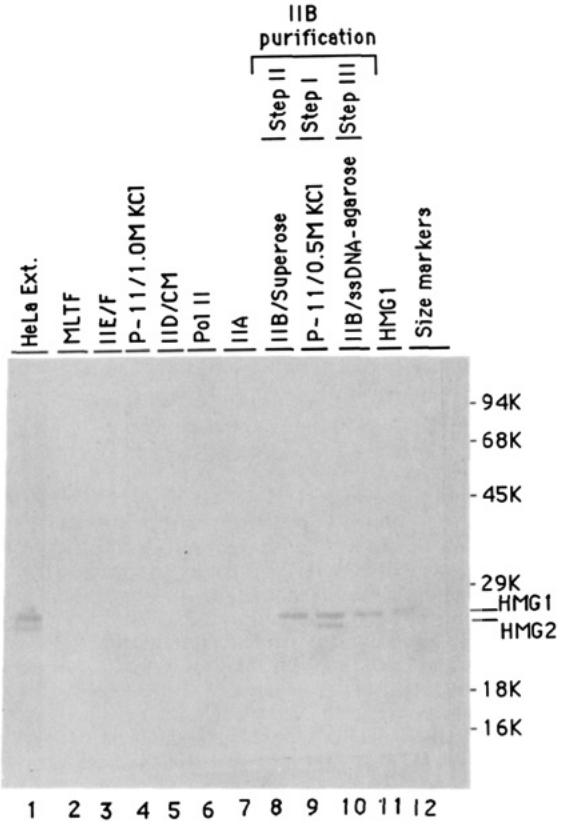

FIGURE 5: HMG antibody cross-reacts with proteins in factor II B preparations. Protein fractions were resolved on a 10% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane, and reacted with HMG antibody as shown in the legend to Figure 1C,D. Lane 1, 10 μL of HeLa nuclear extract; land 2, 5 μL of MLTF; lane 3, 10 μL of TF II E/F; lane 4, 10 μL of the 1.0 M KCl wash fraction of phosphocellulose P11; lane 5, 10 μL of the TF II D/CM fraction; lane 6, 5 μL of the RNA pol II fraction; lane 7, 10 μL of the 0.1 M KCl wash fraction of phosphocellulose P-11 (TF IIA); lane 8, 0.5 M KCl wash fraction of phosphocellulose P-11 (TFIIB); lane 9, 10 μL of the Superose-sizing column fraction (TFIIB); lane 10; 10 μL of the ssDNA–agarose column/0.25 M KCl wash fraction; lane 11, 200 ng of calf thymus HMG-1; lane 12, size markers.

These results show that HMG-1 and -2 copurify with factor IIB up to the step of purification on the ssDNA–agarose column. To test whether HMG-1 and -2 are functionally identical with factor IIB, an experiment was designed to check if the HMG antibody could inhibit the in vitro transcription of the $ML(C_2AT)_{19}$ template using the purified transcription factors (Sawadogo & Roeder, 1985) and whether factor IIB could relieve this inhibition. Increasing amounts of HMG antibody were first preincubated with three different concentrations of factor IIB (the Superose fraction) at 4 °C, and then the remaining factors, the template, and the nucleotides were added and incubated in a transcription reaction. As shown in Figure 6, increasing amounts of HMG antibody caused a progressive inhibition of specific transcription (compare lanes 3, 6, 9, and 12 with lane 2, Figure 6). One microgram of HMG antibody produced complete inhibition (lane 12), while even 9 μg of preimmune IgG had no effect (lane 15). When increasing amounts of factor IIB were preincubated with the antibody before addition of the remaining

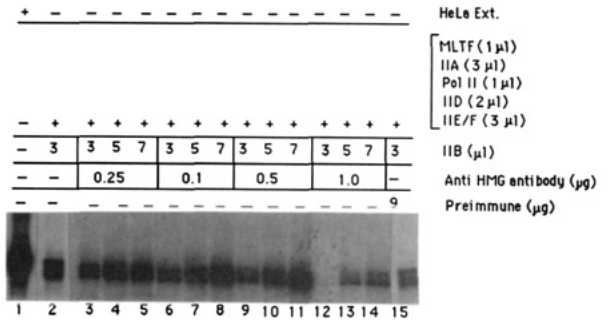

FIGURE 6: Inhibition of reconstituted transcription of MLP$(C_2AT)_{19}$ by HMG antibody and restoration by excess of TFIIB (sizing column fraction). 3, 5, and 7 μL of TFIIB (0.14 μg/μL) were incubated with 0.025 μg (lanes 3–5), 0.1 μg (lanes 6–8), 0.5 μg (lanes 9–11), and 1 μg (lanes 12–14) and HMG antibody or 9 μg of preimmune IgG (lane 15) in the transcription buffer at 4 °C for 30 min; lane 2, complete reaction; lanes 3, 6, 9, and 12, 3 μL of TFIIB; lanes 4, 7, 10, and 13, 5 μL of TFIIB, and lanes 5, 8, 11, and 14, 7 μL of TFIIB were used. Lane 1, 13 μL of extract alone. After the incubation, the remaining transcription factors, the template DNA, and nucleotides (6 mM ATP, 6 mM CTP, 0.125 mM UTP, and 5 μCi of [α-^{32}P]UTP) were added, and incubation carried out at 30 °C for 60 min. Further processing of reaction mixtures was as given in the legend to Figure 2. The reaction volume was 40 μL.

factors, the template pML$(C_2AT)_{19}$, and the nucleotides, a progressive restoration of transcription was observed at each antibody concentration (lanes 4, 5, 7, 8, 10, 11, 13, and 14, Figure 6), and at the inhibiting antibody concentration (lane 12), up to 50% restoration of transcription was observed at the highest concentration of factor IIB used (lane 14, Figure 6).

These results demonstrate that HMG-1 and -2 do copurify functionally with factor IIB, which, together with the observation that HMG-1 and -2 could replace the DE-52/0.15 M KCl flowthrough fraction in complementing transcription by the DE-52/0.15–0.5 M KCl eluate fraction (Figure 4A,B), provide positive evidence for the function of HMG-1 and -2 as general transcription factors for class II genes. Since the factor IIB preparation used in these experiments was not purified to homogeneity, it cannot be unequivocally stated that HMG-1 and -2 are the same as factor IIB. Therefore, we think that HMG-1 and -2 are either identical with factor IIB or are tightly associated with factor IIB in a complex. The former possibility could not be tested because of nonavailability of the previously described factor IIB that migrates as a 2.8S fraction on glycerol gradients and runs as a doublet on SDS–polyacrylamide gels (Reinberg & Roeder, 1987).

Some of the known properties of HMG-1 and -2 need to be mentioned, i.e., the presence of a carboxyl-terminal polyacidic domain of 30 residues consisting exclusively of Glu and Asp residues (Pentecost & Dixon, 1984; Pentecost et al., 1985; Lee et al., 1987) and their ability to unwind DNA and to bind to specific DNA sequences (Wright & Dixon, 1988). The acidic domains, also referred to as the "acid blobs" (Sigler, 1988) present in several known transcription factors (Earnshaw, 1987; Ma & Prashne, 1987), have been shown to be important for the gene activation function of the transcription factors (Earnshaw, 1987).

In the case of HMG-1 and -2, the acidic domains may interact with histones through electrostatic interactions [as shown by in vitro cross-linking studies (Bernues et al., 1986; Carballo et al., 1983)] and unfold the nucleosomes, or they may be involved in interactions with the C-terminal heptad repeat of RNA polymerase II in a fashion similar to that suggested by Sigler (1988), i.e., through electrostatic interactions and/or hydrogen binding. Our preliminary data also suggest an interaction between HMG-1 and -2 and calf thymus RNA polymerase II. The remainder of the HMG molecule may also be responsible for DNA unwinding (Carballo et al., 1983), a proiperty that is likely to be important in changing the topology of the active chromatin.

Added in Proof

By use of a novel band-shift assay in agarose gels, HeLa nuclear extract, that had been immunodepleted with anti-HMG antibody, was found to be defective in the assembly of a heparin-resistant complex with thymidine kinase promoter; addition of HMG 1 could greatly restore complex formation (unpublished experiments).

Acknowledgments

We thank Dr. David P. Bazett-Jones for the gift of human β-globin clone (pβBam) and Dr. J. Jankowski for the trout protamine pJP22, HSV-1 thymidine kinase (pMWTK), and Ad2 MLP pSmaF genes. The calf thymus HMG-1 used in Figure 4B was a gift of Dr. G. R. Reeck, Department of Biochemistry, University of Kansas. Technical help from Manfred Herfort is gratefully acknowledged. We also thank Danny Reinberg for supplying us with enriched or purified transcription factors and the pML $(C_2AT)_{19}$ template.

References

Abdul-Razzak, K. K., Garg, L., Wen, L., & Reeck, G. R. (1987) *Prep. Biochem. 17*, 51–61.
Bucci, L. R., Brock, W. A., Goldknopf, I. L., & Meistrich, M. L. (1984) *J. Biol. Chem. 259*, 8840–8846.
Burton, Z. F., Killeen, M., Sopta, M., Ortolan, L. G., & Greenblatt, J. (1988) *Mol. Cell. Biol. 8*, 1602–1613.
Bernues, J., Espel, E., & Guerol, E. (1986) *Biochim. Biophys. Acta 866*, 242–251.
Bustin, M., & Neihart, N. K. (1979) *Cell 16*, 181–189.
Carballo, M., Puigdomenech, P., & Palau, J. (1983) *EMBO J. 2*, 1759–1764.
Dignam, J. D., Martin, P. L., Shastry, B. S., & Roeder, R. G. (1983) *Methods Enzymol. 101*, 582–588.
Dorbic, T., & Wittig, B. (1987) *EMBO J. 6*, 2393–2399.
Duguet, M., & DeRecondo, A. M. (1978) *J. Biol. Chem. 253*, 1660–1666.
Duguet, M., Soussi, T., Rossignol, J. M., Mechali, M., & DeRecondo, A. M. (1977) *FEBS Lett. 79*, 160–164.
Earnshaw, W. C. (1987) *J. Cell Biol. 105*, 1479–1482.
Flores, O., Maldonado, E., Burton, Z., Greenblatt, J., & Reinberg, D. (1988) *J. Biol. Chem. 263*, 10812–10816.
Gill, G., & Ptashne, M. (1988) *Nature 334*, 721–724.
Haggren, W., & Kolodrubetz, D. (1988) *Mol. Cell. Biol. 8*, 1282–1289.
Hutcheon, T., Dixon, G. H., & Levy, W. B. (1980) *J. Biol. Chem. 255*, 681–685.
Isackson, P. J., Fishbank, J. L., Bidney, D. L., & Reeck, G. R. (1979) *J. Biol. Chem. 254*, 5569–5572.
Isackson, P. J., Debold, W. A., & Reeck, G. R. (1980) *FEBS Lett. 119*, 337–342.
Jackson, J. B., Pollock, J. M., Jr., & Rill, R. L. (1979) *Biochemistry 18*, 3739–3748.
Jankowski, J. M., & Dixon, G. H. (1984) *Can. J. Biochem. Cell Biol. 62*, 291–300.
Javaherian, K., Liu, L. F., & Wang, J. C. (1978) *Science 199*, 1345–1346.
Javaherian, K., Sadeghi, M., & Liu, L. F. (1979) *Nucleic Acids Res. 6*, 3569–3580.
Johns, E. W. (1982) in *The HMG Chromosomal Proteins* (Johns, E. W., Ed.) pp 1–7, Academic Press, New York.

Jones, K. A., Yamamoto, K. R., & Tjian, R. (1985) *Cell 42*, 559–572.
Kleinschmidt, J. A., Scheer, V., Dehauvalle, M.-C., Bustin, M., & Franke, W. W. (1983) *J. Cell Biol. 97*, 838–848.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lee, K.-L. D., Pentecost, B. T., D'Anna, J. A., Tobey, R. A., Gurley, L. R., & Dixon, G. H. (1987) *Nucleic Acids Res. 15*, 5051–5068.
Levy, W. B., Wong, N. C. W., Watson, D. C., Peters, E. H., & Dixon, G. H. (1977a) *Cold Spring Harbor Symp. Quant. Biol. 42*, 793–801.
Levy, W. B., Wong, N. C. W., & Dixon, G. H. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2810–2814.
Levy, W. B., Connor, W., & Dixon, G. H. (1977c) *J. Biol. Chem. 254*, 609–620.
Ma, J., & Pteshne, M. (1987) *Cell 50*, 137–142.
Mardian, J. W. K., Paton, A. E., Bunick, G. H., & Olins, D. E. (1980) *Science 208*, 1534–1536.
Mayes, E. L. W. (1982) in *The HMG Chromosomal Proteins* (Johns, E. W., Ed.) pp 9–40, Academic Press, New York.
McKnight, S. L. (1982) *Cell 31*, 355–365.
Nicolas, R. H., & Goodwin, G. H. (1982) in *The HMG Chromosomal Proteins* (Johns, E. W., Ed.) pp 41–68, Academic Press, New York.
Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–348.
Pentecost, B., & Dixon, G. H. (1984) *Biosci. Rep. 4*, 49–57.
Pentecost, B., Wright, J. M., & Dixon, G. H. (1985) *Nucleic Acids Res. 13*, 4871–4888.
Reinberg, D., & Roeder, R. G. (1987) *J. Biol. Chem. 262*, 3310–3321.
Sawadogo, M. (1988) *J. Biol. Chem. 263*, 11994–12001.
Sawadogo, M., & Roeder, R. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4394–4398.
Seyedin, S. M., & Kistler, W. S. (1979) *J. Biol. Chem. 254*, 11264–11271.
Sigler, P. B. (1988) *Nature 333*, 210–212.
Stoute, J. A., & Marzluff, W. F. (1982) *Biochem. Biophys. Res. Commun. 107*, 1279.
Tremethick, D. J., & Molloy, P. L. (1986) *J. Biol. Chem. 261*, 6986–6992.
Vidali, G., Boffa, L. C., & Allfrey, V. G. (1977) *Cell 12*, 409–415.
Wagner, M. J., Sharp, J. A., & Summers, W. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1441–1445.
Weintraub, H., & Groudine, M. (1976) *Science 193*, 848–853.
Weisbrod, S., & Weintraub, H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 630–634.
Weisbrod, S., Groudine, M., & Weintraub, H. (1980) *Cell. 19*, 289–301.
Wright, J. M., & Dixon, G. H. (1988) *Biochemistry 27*, 576–581.