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Extraction and Partial Characterization of Non-Histone Nuclear Proteins of *Schistosoma mansoni*

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Abstract A pool of nuclear proteins from adult worms of *Schistosoma mansoni* was analyzed for amino acid composition and found to be compatible with high mobility group (HMG) proteins. One of the schistosome HMG proteins was identified as HMG 2 by one-dimensional and two-dimensional PAGE. Stage-specific differences in the HMG-like protein composition were encountered when adult worms were compared to schistosomula, the larval form. Immobilization of the adult male and female nuclear proteins onto nitrocellulose, followed by hybridization against ^{32}P-F-10, a schistosome sex specific gene encoding a major egg shell protein, revealed distinct banding patterns. On the other hand, a synthetic oligonucleotide, derived from the 3'untranslated end of the F-10 gene and possibly containing one regulatory element of the gene, bound mainly to male low MW proteins. © 1992 Wiley-Liss, Inc.

Key words: HMG proteins, *Schistosoma mansoni*, sex-specific gene, DNA binding, regulation

Gene expression in eukaryotes is subordinated to regulation at several levels, from the DNA molecule to the gene product itself. At the level of transcriptional control proteins associated with the chromatin play a major role in dictating when and how frequently a specific gene will direct RNA synthesis.

A group of nuclear proteins, the high mobility group proteins (HMG), first described by Goodwin et al. [1973], besides contributing towards the structural organization of the chromatin internucleosomal regions, appears to participate directly in the regulation of genome expression. The HMG proteins are present in the nucleus in relatively high concentrations and are usually associated with actively transcribing genes. This is particularly evident for HMG 14 and HMG 17 [Bustin et al., 1990]. Among the regulatory proteins that share structural motifs with HMG proteins we can include the RNA polymerase I transcription factor [Bachvarov and Moss, 1991], and the sex determining gene product in humans [Sinclair et al., 1990].

The mechanism through which the HMGs control transcription is still speculative. Results showing in vitro inhibition of deacetylase enzymes by HMG 14 and HMG 17 [Sterner et al., 1981] and also employing specific antibody microinjection, suggest that the regulatory properties of these proteins might be related to their ability to block or unblock radicals responsible for the binding of the ligand to DNA. Binding of HMG 14 to nucleosomes is also affected by their degree of phosphorylation as recently reported [Spaulding et al., 1991].

Schistosoma mansoni, the causative agents of schistosomiasis, which affects millions of people in Africa and South America, unlike most digenetic trematodes, are dioecious parasites; that is, the individuals differentiate sexually. Adults display a clear sexual dimorphism, but schistosomula, the larval stage, can only be distinguished at the molecular level, through the detection of satellite DNA sequences present in the heterochromatin of the W chromosome of female parasites [Walker et al., 1989]. Because of the intrin-

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sic interest in understanding the biochemistry of sexual differentiation in these helminths, research on the molecular aspects of this process has begun to attract the attention of some groups. Thus, the search for differences in protein composition of the various developmental stages of schistosomes has been undertaken [Yuckemberg et al., 1987; Braga et al., 1989]. Likewise, maturation linked and sex-specific genes, which are only transcribed in adult females and which encode major egg shell proteins, have already been characterized and sequenced [Bobeck et al., 1986; Simpson et al., 1987].

In spite of these efforts, little has been learned about the mechanisms of regulation of development per se. As mentioned above, nuclear proteins are believed to play a key role in the regulation of transcription. It would thus be desirable to obtain information on the protein composition of the schistosome chromatin, a yet unexplored approach.

In the present work we report the composition of the non-histone nuclear proteins of adult male and female worms and schistosomula of *Schistosoma mansoni.* Furthermore, we have extended our observations to include investigations on the potential regulatory roles of the schistosome non-histone nuclear proteins, by analysing their binding characteristics to the sex- and stage-specific gene F-10, expressed only in mature females and to a synthetic oligonucleotide derived from the 3′ end of the F-10 gene bearing a sequence typical of steroid hormone regulatory elements [Beato et al., 1987; Rumjanek et al., 1989].

MATERIALS AND METHODS

Parasites

Adult worms of *Schistosoma mansoni,* LE strain, referred to as bisexual parasites, were obtained by perfusion of Swiss mice according to the method of Smithers and Terry [1965]. Immature male and female worms, referred to as unisexual parasites, were obtained by infecting snails with a single miracidium. Schistosomula were prepared using the technique of Ramalho-Pinto et al. [1974].

Preparation of Nuclear Proteins

Nuclei were prepared essentially as described by Elton and Reeves [1985]. The nuclei were suspended in 4 ml of NaCl 0.35 M, 10 mM Tris buffer pH 7.2 and 1 mM of the protease inhibitor phenylmethanesulphonyl fluoride (PMSF). The nucleoproteins were extracted after lysis of the nuclei using a Sorvall Omnimixer for 2 min at maximum speed. Occasionally, nuclei were lysed by ultrasonication with several short bursts in a Branson sonicator, at low temperature. The homogenate was transferred to a closed tube and agitated at 4°C with a magnetic stirrer for 45 min. The suspension was then centrifuged at 7,000*g* for 15 min in a Sorvall refrigerated centrifuge using the SS 34 rotor and the supernatant saved. The pellet was reextracted as above. To the supernatant two volumes of cold acetone were added and left standing overnight at 4°C. After centrifugation at 8,000*g* for 15 min, a pellet was obtained. This was washed twice with ice cold acetone, dried under vacuum and stored at −70°C until used.

Polyacrylamide Electrophoresis

For electrophoresis, the proteins above were dissolved in 0.9M acetic acid, 4M urea, 1% 2-mercaptoethanol, and 3.5% methyl green. Proteins were fractionated in either 12.5% or 15% polyacrylamide gels, in 4M urea, 0.1% sodium dodecyl sulfate (SDS). Before protein fractionation, the gels had been pre-electrophoresed for 2 h. Electrophoresis was carried out at 60–80 V until the methyl green dye had reached the bottom of the gel. Fractionation of nuclear proteins on 2-D gels was carried out according to O'Farrel [1975]. Proteins were visualized by either Coomassie blue or silver staining. HMG standards were a kind gift from Dr. G. Goodwin from The Chester Beatty Institute. Protein was assayed by the method of Lowry et al. [1951].

DNA Binding to Nuclear Proteins

Characterization of the DNA binding proteins was carried out using the south-western protein blotting method of Bowen et al. [1980]. Fractionation of the male schistosome non-histone nuclear proteins by hydroxyapatite chromatography was done essentially as described by Bloom and Anderson [1978]. The several fractions from the hydroxyapatite column were further separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) using an 8–25% linear acrylamide gradient and transferred electrophoretically to nitrocellulose (Scleicher & Schuell).

Radioactive Labeling of DNA

The F-10 gene was labelled with ^{32}P using the nick translation method [Rigby et al., 1977]. The synthetic oligonucleotide with the sequence, 5'TCTCCACTGTCCTATTTTT 3', [Rumjanek et al., 1989], was end-labelled with ^{32}P using T4 polynucleotide kinase (Boehringer Manheim) according to the manufacturer's recommendations.

Amino Acid Composition

Amino acid composition was determined out after acid hydrolysis of the nuclear extract in 6N HCl under vacuum at 106 C for 20 hs, in a Beckman model 120 C amino acid analyzer.

RESULTS

The results of amino acid composition analysis of the pool of non-histone nuclear proteins of adult schistosomes is shown in Table I. The results show the characteristic large number of basic and acidic amino acids of HMG proteins, with glutamic acid displaying the highest concentration. With the exception of lysine residues, the pattern obtained followed very closely that described for calf thymus [Goodwin et al., 1973].

Figure 1 shows the results of experiments in which schistosome nuclear proteins extractable by 0.35 M NaCl and acetone insoluble were fractionated in 12.5% urea/SDS PAGE. The patterns obtained were similar to these described for other organisms [Elton and Reeves, 1985] and are compatible with the occurrence of several HMGs. The intensely stained doublet in Figure 1 indicated by arrow 1 was found to electrophorese to a region where, normally, in the case of mammalian proteins, HMG 1, 2, and 3 would be detected. The other two bands migrating faster (arrow 2), might correspond to HMG 14 and 17, respectively. Schistosomula, on the other hand, reproducibly displayed a protein band indicated by arrow 3, not seen in adult parasites. The schistosomular nuclear protein is also evident in the 2-D PAGE shown in Figure 1B. This is possible an altogether different HMG-like protein, and its presence in the larval forms may reflect the differences in the biosynthetic status of the individual chromatins.

In adult schistosomes and schistosomula, the high MW band, indicated by arrow 1 in Figure 1, was shown to comigrate with HMG 2 as depicted by the one-dimensional PAGE in Figure 2 and the 2D-gels in Figure 3A,B. The typical pattern of HMG 2, displaying 4 subfractions, is shown in Figure 3A [Walker et al., 1976]. The same subfractions can also be observed among the schistosome high MW nuclear proteins shown in Figure 3B.

Preliminary attempts towards identifying which among the schistosome non-histone nuclear proteins were interacting with the gender specific gene F-10 employed the "South-Western" protein blotting technique. In these experiments the fractionated nuclear proteins were transferred electrophoretically to nitrocel-

TABLE I. Amino Acid Composition of *Schistosoma mansoni* Nuclear Proteins*

Amino acid	μmol/mg protein
W	0.46
K	1.16
H	0.30
R	1.49
D	2.15
T	1.15
S	1.27
E	3.27
P	0.72
G	2.60
A	2.05
V	1.63
M	0.54
I	1.30
L	2.02
Y	0.53
F	0.65
C	—

*Amino acid composition of adult schistosome nuclear proteins. A pool of nuclear proteins (male and female) was extracted and analyzed for amino acid composition after acid hydrolysis as described in Materials and Methods. The data correspond to a sample having a total of 240 μg protein.

Fig. 1. Fractionation of schistosome non-histone nuclear proteins in polyacrylamide-urea gels. **a:** Proteins were extracted from schistosomes according to Materials and Methods, fractionated on 12.5% polyacrylamide/urea gels, and the gels were silver stained. Lanes A–D correspond to 30 μg protein mixture consisting of mature male and female nuclear proteins, 15 μg of mature male, 15 μg of mature female worms, and 10 μg of schistosomula, respectively. Arrows 1–3 point to HMG 2, the low MW HMG-like proteins, and the schistosomular HMG-like protein, respectively. **b:** 150 μg of schistosomular nuclear proteins fractionated on a 2-D PAGE system according to methods. The arrow points to the schistosomular HMG-like protein.

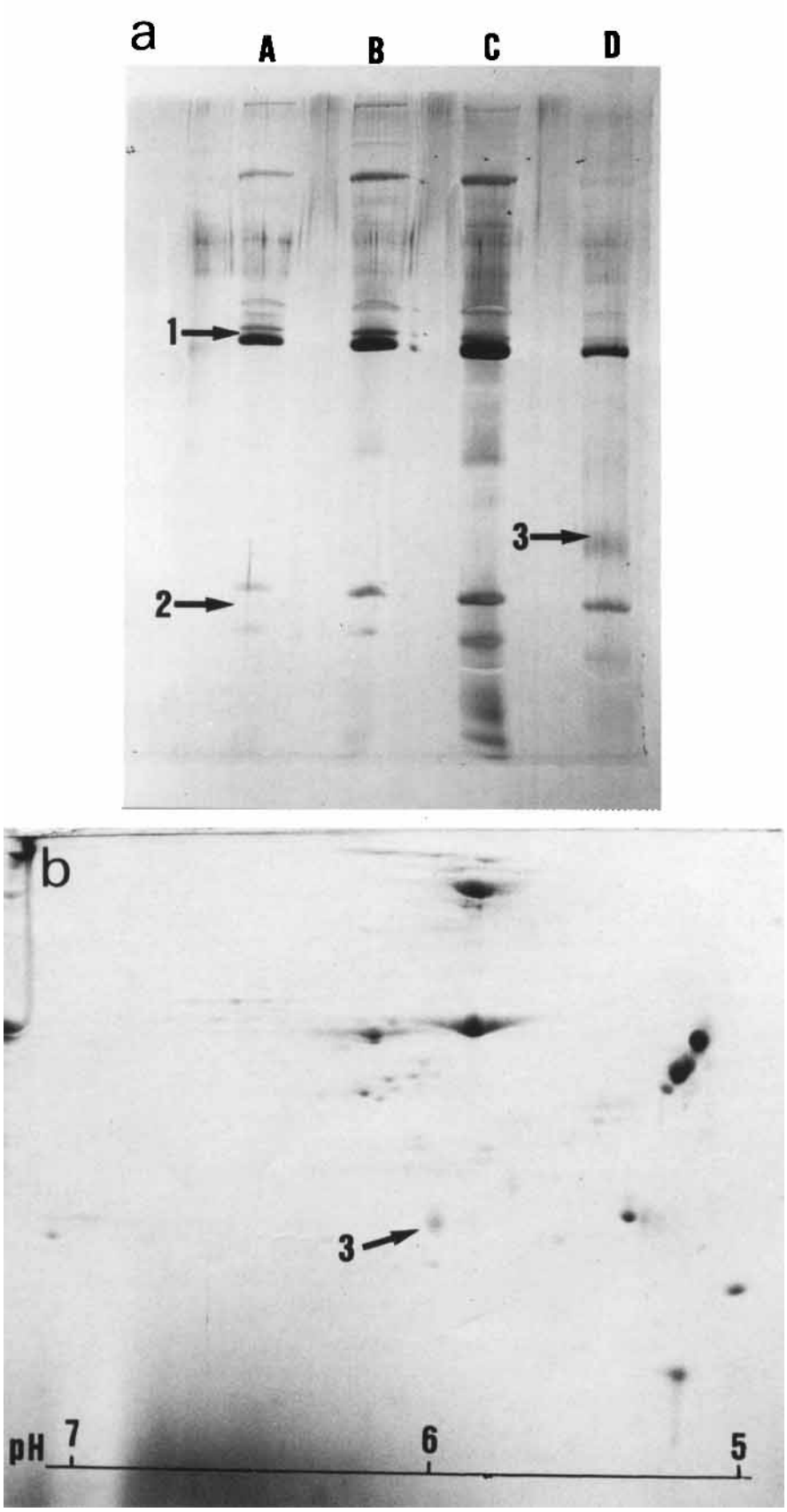

Figure 1.

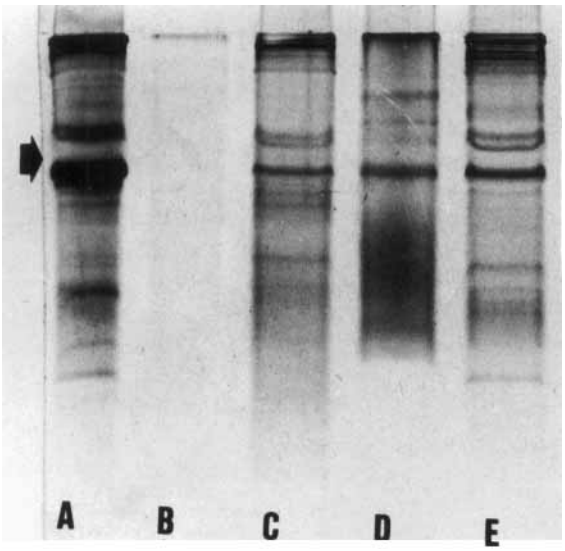

Fig. 2. Fractionation of schistosome non-histone nuclear proteins on 12.5% polyacrylamide/urea gel: comparison with HMG 2 standard. Proteins from schistosomes were extracted as in Materials and Methods and stained with Coomassie blue. Lanes A–E correspond to: 15 μg protein of mature male schistosomes, 10 μg total HMG standards, 15 μg of mature female proteins, 10 μg of HMG 2 standard, and 15 μg of schistosomular proteins, respectively. The arrow indicates the position of HMG 2.

lulose and subsequently hybridized against the radioactive probes.

Figure 4 shows the results of experiments in which male and female nuclear proteins were hybridized against the F-10 DNA. Both preparations showed three major bands in the low molecular weight region of the gel. Although no qualitative differences in the binding pattern of the probe were observed when mature and immature males were compared, the probe bound to a female protein not observed in males, indicated in lane 1. The two more intense bands in the female protein preparation appeared to comigrate with HMG 14 and 17, respectively. In the male extract the middle band comigrated with HMG 14, but the other two bands could not be identified. The pattern shown in Figure 4 was reproduced in several other experiments.

Similar hybridization experiments were carried out testing the binding of the schistosome nuclear proteins to the HRE containing synthetic oligonucleotide. The results are shown in Figure 5. Qualitative differences were quite extensive when comparing male and female worms. Curiously, only the male protein extract showed any high affinity interaction between proteins and the oligonucleotide probe within the low to middle molecular weight range of the electrophoretogram. In contrast, only the very low MW peptides obtained from mature and immature females bound to the probe with high affinity. Low affinity binding to the probe could be detected in a single discrete band showing faintly in the lanes corresponding to mature and immature females. While these results suggest that qualitative differences reflect individual levels of physiological regulation peculiar to each sex, more detailed mechanistic conclusions will have to wait until a functional assay for the relevant proteins becomes available.

Because oligonucleotide binding occurred mainly to male schistosome nuclear proteins, these were further fractionated on a hydroxyapatite column, blotted onto nitrocellulose and hybridized against the oligonucleotide probe as above.

The results are presented in Figure 6A,B. Elution of the column with a discontinuous concentration gradient of sodium phosphate yielded the pattern shown in Figure 6A. In spite of the complexity still seen in each lane, binding of the labelled synthetic oligonucleotide was resolved to only three low molecular weight peptides, eluted from the column with 0.5M NaCl, as shown in Figure 6B, lane 5. The necessity of fractionating the peptides under denaturing conditions might have restricted the binding of the oligonucleotide to the peptides as a result of partial or total loss of their tertiary structure and hence of the integrity of the DNA binding domains.

DISCUSSION

Although the identification of the schistosome nuclear proteins is still incomplete, the proteins characterized in the present work conform to several of the generally accepted features of HMGs. They can be extracted from the chromatin with 0.35 M NaCl and dilute acids, they have small MW, and they contain unusually high concentrations of charged amino acid residues in their structure. In the case of schistosomes, glutamic acid rather than lysine was found to be the most abundant amino acid. However, until the primary structure of the schistosome nuclear proteins is elucidated, perhaps a more cautious denomination would be "HMG-like" proteins as described from Schulman et al. [1987]. Notwithstanding, at least one of the HMGs from schistosomes could be identified as HMG 2,

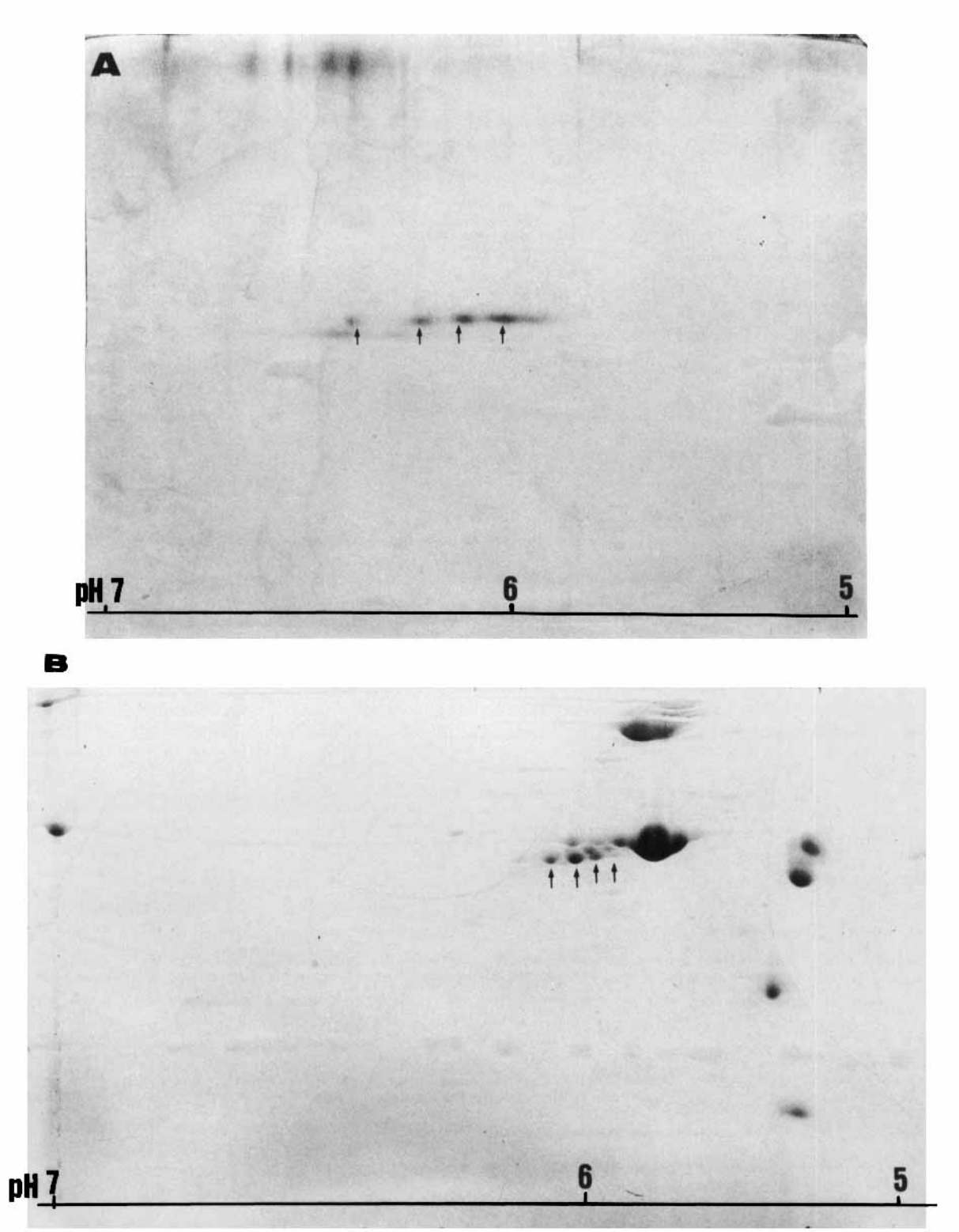

Fig. 3. Fractionation of HMG 2 standard and male schistosome nuclear proteins on 2D gel electrophoresis. **A:** 30 μg of HMG 2 were fractionated as described in Materials and Methods and visualized by silver staining. **B:** 150 μg of male nuclear proteins were fractionated as described in Materials and Methods and visualized by Coomassie blue staining. The horizontal axis indicates the pH range employed. The arrows in A and B indicate the positions of HMG 2.

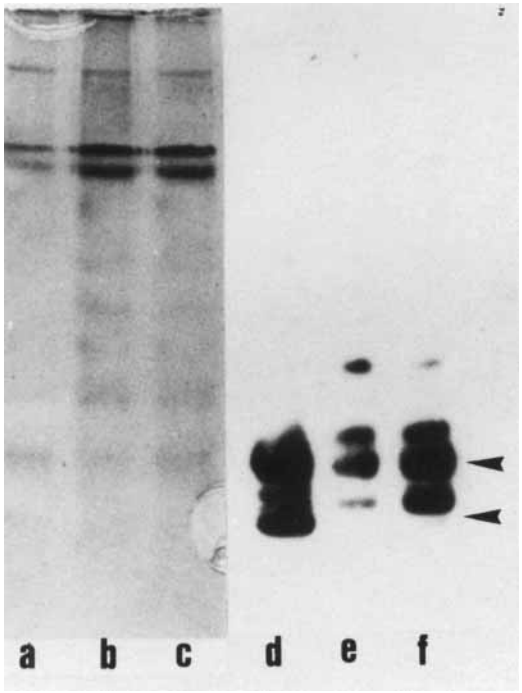

Fig. 4. Hybridization of the ^{32}P-F-10 gene against blotted schistosome nuclear proteins. Nuclear proteins from schistosomes (24 μg were fractionated on 12.5% polyacrylamide/urea gels and transferred to nitrocellulose membranes as described in methods. Lanes a–c correspond to nuclear proteins of mature females, mature males, and immature females, respectively. Lanes d–f correspond to the same material transferred to nitrocellulose and hybridized against the probe, ^{32}P-F-10 (approx. 10^5 cpm). The arrows indicate the position of the low MW HMG-like proteins.

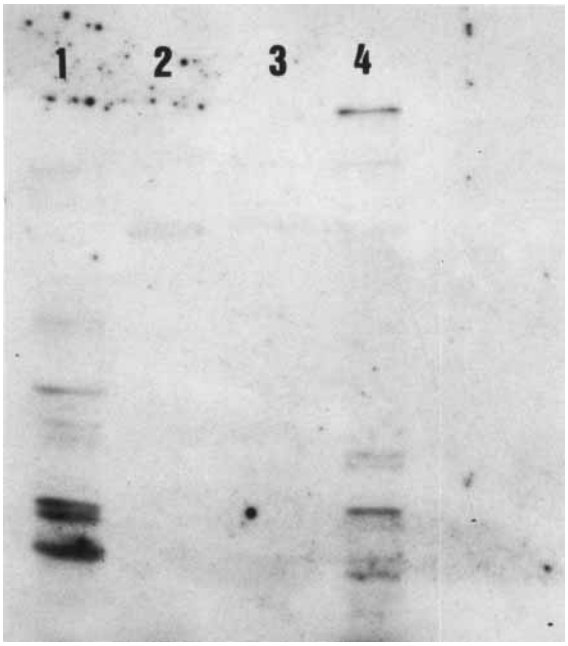

Fig. 5. Hybridization of the ^{32}P-synthetic oligonucleotide against schistosome nuclear proteins. Nuclear proteins from schistosomes were extracted and approximately 20 μg were fractionated on 15% polyacrylamide/urea gels, renatured, and transferred to nitrocellulose membranes as described in Materials and Methods. 8×10^5 cpm of the ^{32}P end-labelled oligonucleotide probe were added to 3 ml hybridization solution and hybridized overnight at room temperature. Lanes 1–4 correspond to mature males, mature females, immature females, and a mixture of mature male and female worms, respectively.

which was found as a major component among the nuclear proteins in adult and larval stages of the parasites. Schistosomula were also shown to contain a nuclear protein not detected in the adult stages. Qualitative differences in the nuclear protein composition of larval and adult stages should not be surprising, however, in view of the observation that newly transformed schistosomula do not exhibit much biosynthetic activity, a situation which would generate a characteristic pattern of regulatory proteins.

That the schistosome HMG-like proteins participate in the regulation of gene expression was also suggested by the results in which the low MW HMG-like proteins were shown, through the southwestern blot technique, to bind to a gender-specific gene and its putative regulatory element. The appearance of a female-specific DNA/protein complex in female schistosome nuclear protein preparations only, may indicate that these HMG-like proteins could be responsible for the activation of transcription of the F-10 gene. Alternatively, the complexes observed in the male protein preparations may represent low MW nuclear proteins which for this particular gene would have a repressor role. "Silencer" proteins, which completely block transcription, are known to occur in yeasts and are probably widespread in eukaryotes [Johnson and McKnight, 1989]. The F-10 gene in male schistosomes may represent a situation in which a negative regulator or silencer protein might have to be invoked. Recent results have shown that indeed, protein preparations from male schistosomes are able to protect the F-10 gene from DNase I digestion more efficiently than female proteins [Engelender and Rumjanek, in press].

In the present work, gender related differences in the binding pattern were also observed when the synthetic oligonucleotide representing a putative HRE of the F-10 gene was used as a probe (Fig. 6). In this instance, binding occurred primarily to several low MW male proteins, which upon purification on hydroxyapatite revealed three protein bands as the major ligands.

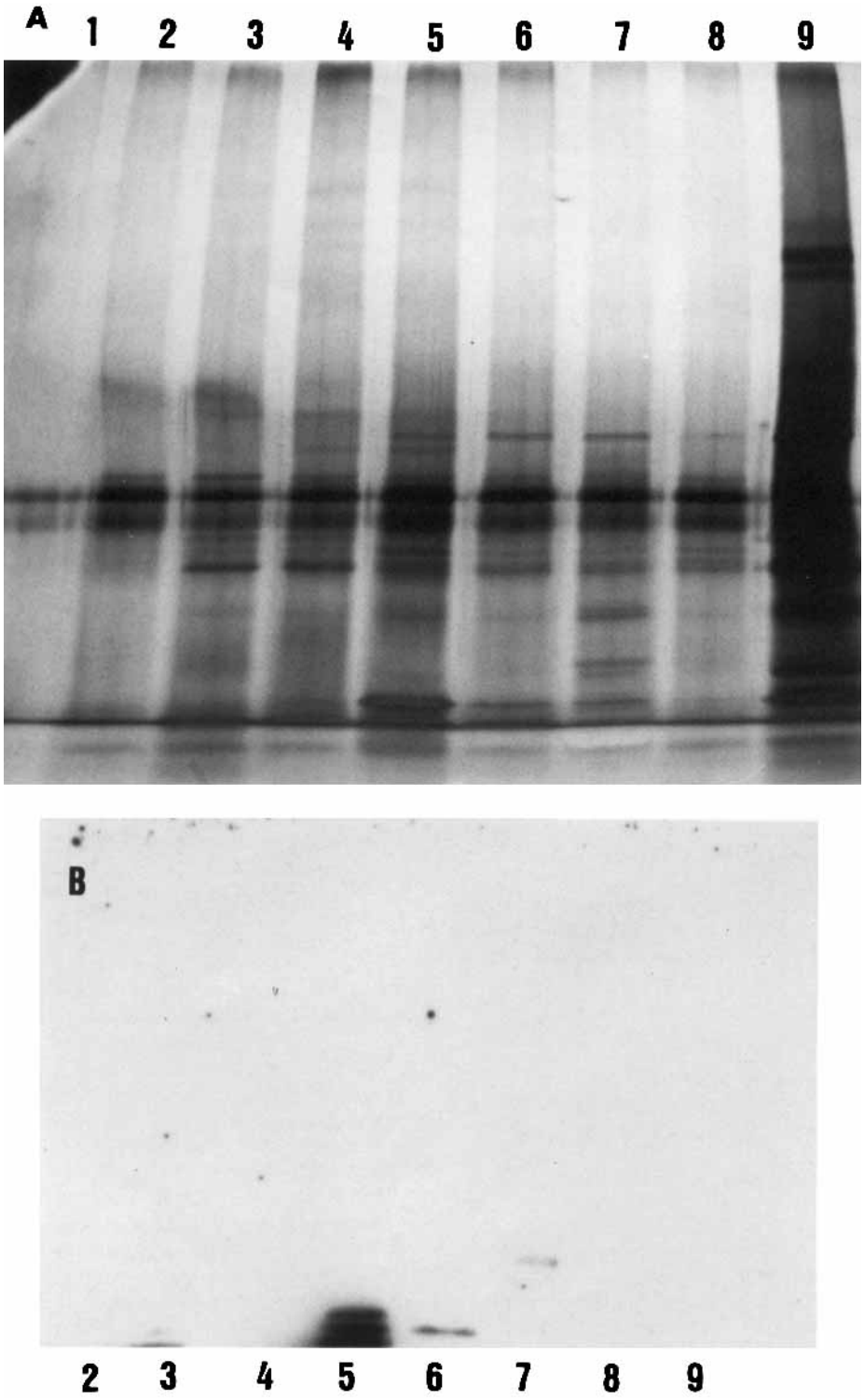

Fig. 6. Hybridization of the ^{32}P-synthetic oligonucleotide against adult male nuclear proteins fractionated on hydroxyapatite and polyacrylamide/urea gels. Nuclear proteins from male schistosomes were extracted and purified on a hydroxyapatite column as described in Materials and Methods. The fractions from the column were then concentrated and applied on 8–25% polyacrylamide gels and transferred to nitrocellulose as described earlier. Hybridization was carried out overnight at room temperature. **A:** Silver stained polyacrylamide gel. Lanes 1–9 correspond to fractions eluted from the hydroxyapatite column with 1 mM NaP, 80 mM NaP, 80 mM NaP + 0.25 M NaCl, 80 mM NaP + 0.5 M NaCl, 80 mM NaP + 0.75 M NaCl, 80 mM NaP + 1.25 M NaCl, 80 mM NaP + 2 M NaCl and 80 mM NaP + 2 M NaCl + 5 M urea, respectively. **B:** Autoradiograph of nitrocellulose membrane containing same fractions as in A and hybridized to the oligonucleotide probe.

It remains to be established whether these proteins have a role as negative modulators of the male F-10 gene. Because the oligonucleotide probe used in the above experiments contains a key regulatory sequence it would be reasonable to assume that proteins with a function of shutting-off transcription would bind to or near the same site interacting with steroid receptors, represented here by the oligonucleotide probe.

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