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H5 Gene-Specific *trans*-Activation by Nuclear Extracts from Avian Erythroid Cells

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Nuclear extracts from chicken erythroid cells selectively stimulate transcription of the chicken histone H5 gene (and not of other chicken histone genes) after coinjection into frog oocytes. This effect is shown to involve an enhancerlike activity, and a region of the H5 gene sufficient to mediate *trans*-activation is defined.

H5 is a linker histone variant (1, 13) which, in the chicken, is found only in erythroid cells (19). Levels of H5 protein increase during the differentiation and maturation of these cells (11, 17, 21), and this increase is correlated with chromatin condensation and repression of replication and transcription (17). We have previously isolated and characterized the single-copy chicken H5 gene (9) and demonstrated that transcription of this gene is accurately initiated in *Xenopus* oocytes (22). Our current interest lies in the transcriptional regulation of this gene.

We report here our use of the frog oocyte to identify factors in erythroid-cell extracts that are involved in transcription of the H5 gene. Coinjection of DNA and cell extracts into frog oocytes has previously been used in the identification of regulatory factors for sea urchin histone genes (5, 12, 20) and in transcriptional studies with the adenovirus E1A protein (7, 15).

Effect of erythroid-cell extracts on the level of H5 transcripts in coinjected oocytes. Chromatin salt-wash fractions (CSWFs) were isolated from a chicken erythroid cell line by the methods of Stunnenberg and Birnstiel (20). The cells used (ts34 AEV LSCC HD3 [2]) (AEV cells) are transformed by a temperature-sensitive avian erythroblastosis virus, and they express H5 mRNA and protein (19).

To test the fractions for possible effects on H5 gene expression, CSWF samples were coinjected, with the H5 gene and control genes, into batches of frog oocytes. The injected DNA was a mixture of pH5/2.6 DNA, containing the H5 gene from −1,200 to +1,360 in pBR322 (Fig. 1a), and pH1/H2B DNA, containing two chicken histone genes, H1 and H2B (from pCH7.0E [3]), in pAT153. As shown in Fig. 2a, the CSWFs were injected into the oocyte cytoplasm, followed by nuclear injection of the DNA mixture. Oocyte manipulations were carried out according to Gurdon (6). DNA (5 to 10 ng) and CWSF (protein) (25 to 100 ng) were injected into each oocyte, and after incubation, total RNA was isolated from pooled batches of 25 oocytes (14).

Figure 2b shows the result of quantitative primer extension analysis (10) of the oocyte RNA with synthetic 26-base primers. Extension on all three histone transcripts was carried out in the same reaction; each transcript generated two or more major extension products (due to cap site heterogeneity). Track 1 shows the result obtained when buffer alone was coinjected with the DNA, and track 2 shows coinjection of an AEV cell CSWF with the DNA.

By comparing the two tracks, it can be seen that injection of the CSWF did not affect the level of H1 and H2B transcripts produced. In contrast, injection of the CSWF resulted in a dramatic increase (of at least 10-fold) in the level of H5 transcripts.

This effect cannot be due to the presence of H5 mRNA in the nuclear extracts, since previous work has shown that the AEV cell H5 mRNA has a 9-base insertion in the 5′ untranslated region, yielding a longer primer extension product than the transcript from the cloned H5 gene (16, 18, 22).

Enhancerlike activity associated with the H5 gene. The H2B gene from pH1/H2B, used as a control in the experiment described above, was cloned next to the H5 gene in an M13 vector. In this construct, named mH5/H2B, the two genes are in a divergent arrangement (Fig. 3a), and the distance between the cap sites of the two genes is approximately 1,300 base pairs (bp).

For CSWF coinjection experiments, mH5/H2B DNA was mixed with pH1/H2A DNA, a construct containing the H1 gene from pH1/H2B and a chicken histone H2A gene (Fig. 3a). Figure 3b presents the results of one such coinjection experiment, obtained by primer extension analysis of oocyte RNA. Batches of 25 oocytes were injected with the mH5/H2B plus pH1/H2A DNA mixture, together with bovine serum albumin as a control (track 1) or with AEV cell CSWFs (tracks 2 to 6). In this and subsequent experiments, the DNA and CSWFs were mixed together and injected into the oocyte nucleus.

By comparing tracks 2 to 6 with track 1, it can be seen that injection of the CSWFs did not significantly increase the level of H1 and H2A transcripts (minor differences in overall transcript levels represent variability in the efficiency of nuclear injection between individual batches of oocytes). As observed in previous experiments (Fig. 2b), injection of the CSWFs results in a large increase in the level of H5 transcripts (relative to H1 and H2A transcripts). However, in this experiment (Fig. 3b), in contrast to the result shown in Fig. 2b, injection of the CSWFs also resulted in a dramatic increase in the level of H2B transcripts. (Interestingly, this increase is reproducibly higher for H2B than for H5, suggesting that H2B is capable of a greater maximal rate of transcription before oocyte factors become limiting.)

Thus, when the H5 and H2B genes were introduced into oocytes on separate plasmids, the CSWFs only produced an increase in the level of H5 transcripts (Fig. 2b), but when the two genes were physically linked, the levels of both gene transcripts were increased (Fig. 3b).

These results indicate that the CSWFs must exert their effect at the transcriptional level, rather than at the level of mRNA stability, since only the former hypothesis is consistent with the result shown in Fig. 3b.

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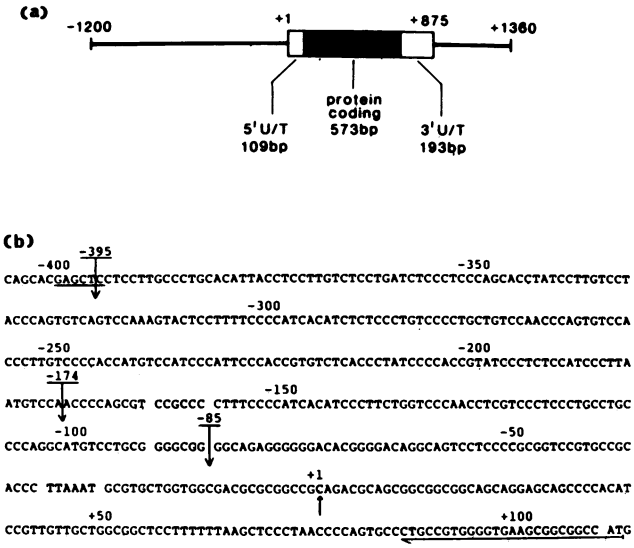

FIG. 1. The chicken histone H5 gene and its 5'-flanking sequence. (a) Schematic representation of the H5 gene in pH5/2.6. The numbers indicate the distance in base pairs from the transcription initiation site (cap site), at +1. U/T, Untranslated region. (b) DNA sequence of a portion of the 5' flanking region of the H5 gene. The TATA box (at −30) and the GC boxes (at −160 and −90) are shown separated from the rest of the sequence. The binding site for the H5 26-base primer is underlined. Downward arrows indicate the locations of the endpoints of the 5' deletions used in the oocyte experiments. The upward arrow indicates the cap site. The numbers directly above the sequence indicate the distance in base pairs from the cap site (+1); the exact base associated with the number is beneath the second digit from the right-hand side (and directly beneath the 1 in the case of the cap site).

This result also suggests that the effect of the CSWFs involves an enhancerlike activity which stimulates transcription of both the H5 and H2B genes on mH5/H2B, since these genes are divergently transcribed and their cap sites are separated by greater than 1,200 bp.

This transcription enhancement must be mediated by H5 gene sequences, and not vector sequences, since *trans*-activation of the H5 gene was obtained with the gene in both a pBR-derived vector (pH5/2.6; Fig. 2b) and an M13-derived vector (mH5/H2B; Fig. 3b). Furthermore, transcription of the H2B gene was enhanced only when it was linked to the H5 gene, and not when it was present in pH1/H2B (Fig. 2b) or when it was injected as a separate M13 clone (data not shown).

Tracks 2 to 6 in Fig. 3b show injection of different CSWFs, isolated by sequential extraction of chromatin with increasing concentrations of NaCl (see legend to Fig. 3). Surprisingly, each CSWF is capable of producing *trans*-activation. The reason for this has not yet been determined, but it is possible that fractionation was inefficient at each step or that the stimulatory factors are present in different forms in the nucleus. Most importantly, this result does not affect any of the conclusions that we have drawn and discussed above.

A region of the H5 gene sufficient to mediate *trans*-activation. An initial investigation was undertaken to identify the region(s) of the H5 gene involved in the transcription stimulation produced by the CSWFs. In this study, gross deletions of the H5 gene were used in CSWF coinjection experiments.

First, constructs containing the entire transcribed region of the H5 gene but with large deletions of 5'-flanking region were tested for their response to CSWFs, following coinjection into oocytes. The H5 gene in pH5/2.6 and mH5/H2B, used in previous experiments, contained approximately 1,200 bp upstream of the cap site. The two 5'-deletion constructs tested, mH5/−174 and mH5/−85, had only 174 and 85 bp upstream of the cap site, respectively (Fig. 1b). Full levels of *trans*-activation were produced with both of these deletions, and the result obtained with mH5/−85 is shown in Fig. 4b.

To further define the region involved in *trans*-activation, a large 3' deletion of the H5 gene was constructed. This clone, mH5/−174:+313, was derived from mH5/−174 and contains the H5 gene from −174 to +313 (relative to the cap site at +1 [Fig. 4a]). This construct was tested in the oocyte coin-

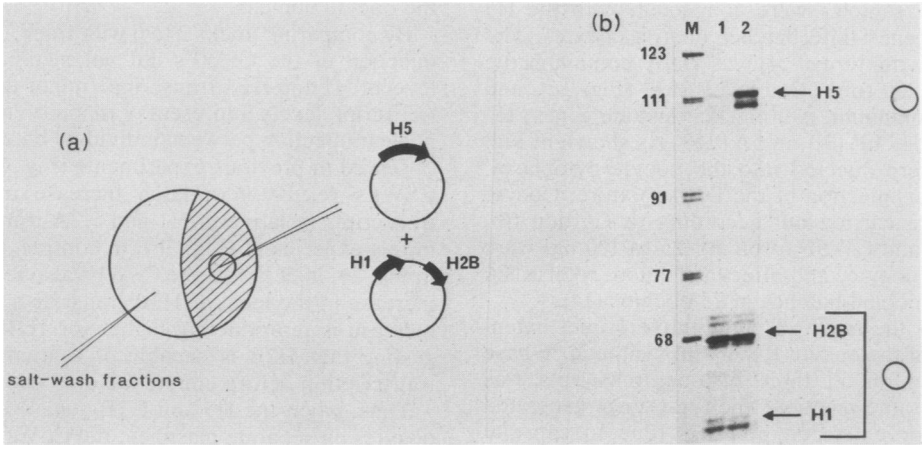

FIG. 2. (a) Diagrammatic representation of the experiment used to test the effect of the AEV cell CSWFs on H5 gene transcription in coinjected frog oocytes. The two plasmids shown represent pH5/2.6 and pH1/H2B. (b) Result of quantitative primer extension analysis of total RNA isolated from oocytes coinjected as shown in panel a. Analysis was carried out using the synthetic 26-base primers. M is a marker track, in which end-labeled *Hpa*II-cut pBR322 DNA was run; sizes are shown in base pairs. Track 1 shows injection of the CSWF storage (and injection) buffer with the DNA mixture, as a control. Track 2 shows injection of an AEV cell CSWF with the same DNA mixture. The H5, H2B, and H1 extension products are indicated. The square bracket and circles emphasize that the H1 and H2B genes were linked on the same plasmid, separate from the plasmid containing the H5 gene. An amount of RNA equivalent to that from one oocyte was used in each extension reaction, and the extension products were electrophoresed on a standard 6% polyacrylamide sequencing gel.

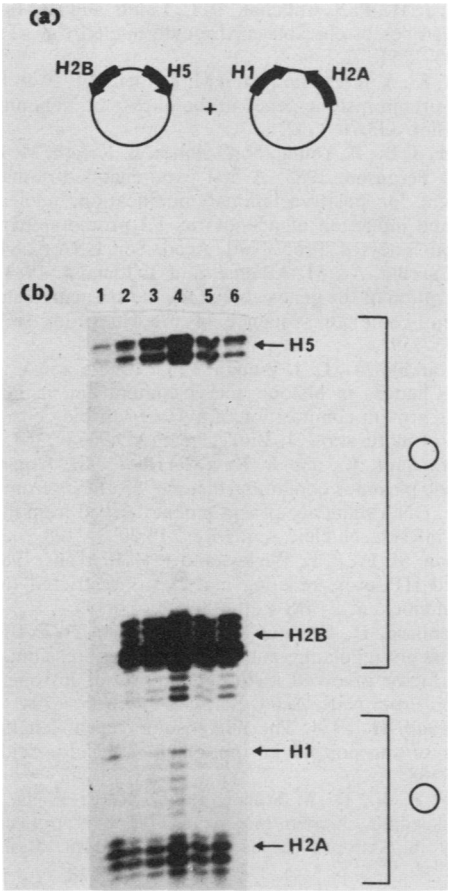

FIG. 3. (a) Diagram representing the plasmids mH5/H2B and pH1/H2A. The direction of transcription of the genes on these plasmids is indicated. (b) Result of quantitative primer extension analysis of total RNA isolated from coinjected oocytes. Track 1 shows injection of bovine serum albumin, with a mixture of mH5/H2B and pH1/H2A DNA, as a control. Tracks 2 to 6 show injection of AEV cell CSWFs with the same DNA mixture. The CSWFs were extracted sequentially with 150 mM (track 6), 300 mM (track 5), 450 mM (track 4), 600 mM (track 3), and 2 M NaCl (track 2). The H5, H2B, H1, and H2A extension products are indicated. The square brackets and circles emphasize the gene content of the two plasmids used in this experiment. An amount of RNA equivalent to that from one oocyte was used in each extension reaction.

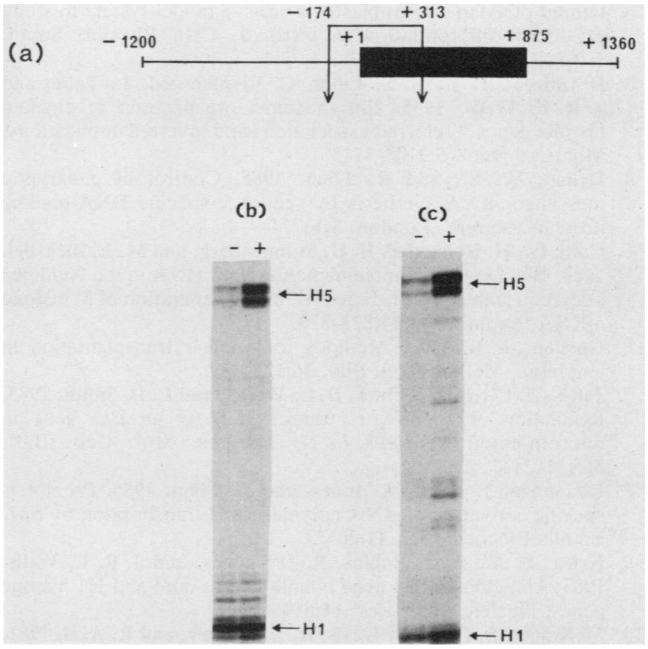

FIG. 4. (a) A diagram showing the endpoints of the H5 gene in the construct mH5/−174:+313, −174 and +313 (bp), relative to the cap site at +1. The line diagram represents the H5 gene in pH5/2.6 (as shown in Fig. 1a). Panels b and c both show the result of quantitative primer extension analysis of RNA isolated from oocytes coinjected with bovine serum albumin (−), as a control, and with an AEV cell CSWF (+). The coinjected DNA mixture consisted of mH5/−85 and pH1/H2B (b) and mH5/−174:+313 and pH1/H2A (c). The H5 and H1 extension products are indicated. The H2B and H2A transcripts showed the same lack of response to CSWF injection as H1 transcripts (data not shown).

jection assay and showed full levels of *trans*-activation (Fig. 4c).

The results obtained with the H5 gene deletion constructs suggest that the 398-bp region of the gene between −85 and +313 is sufficient to mediate the *trans*-activation produced by the AEV cell CSWFs.

Oocyte injection experiments done in the absence of CSWFs (M. F. Shannon, P. L. Wigley, A. J. Robins, and J. R. E. Wells, manuscript submitted) have defined two transcription modulator regions upstream of the H5 gene. The region between −395 and −174 exerted an inhibitory effect on H5 transcription, while the region from −174 to −85 had a stimulatory effect (see Fig. 1b for the location of these regions). It is likely that the two sequences in this latter region with homology to the GC box (4, 8; Fig. 1b) are responsible for the positive effect of this domain on H5 transcription. The finding that sequences upstream from −85 are not necessary for the *trans*-activation reported here indicates that the effect of the AEV cell CSWFs is not the result of relieving the inhibitory effect of the −395 to −174 region, nor can it be explained by the binding of Sp1 (4, 8; possibly present in the CSWFs) to the two GC boxes located 5′ to −85. In addition, no specific sequences which have previously been demonstrated to mediate enhancement of transcription are located in the region from −85 to +313 of the H5 gene.

In summary, we have identified one or more factors, present in chicken erythroid-cell nuclei, which are involved in the stimulation of H5 gene transcription. The work presented here is currently being extended to purify and characterize the specific factors involved in *trans*-activation, to investigate their interaction with the H5 gene, and to determine their cell type specificity.

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