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Basal expression of the histone H5 gene is controlled by positive and negative *cis*-acting sequences

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ABSTRACT

Sequences from -3500 to +1365 of the chicken histone H5 gene have been analyzed for the presence of cis-acting elements in H5 expressing (transformed CFU-E) and non-expressing cells (fibroblasts). The region from -3500 to -115 had little effect on transcription. Proximal upstream sequences contain a negative element (UNE, -115 to -95), capable to also repress the activity of the heterologous HSV tk promoter, and two positive elements, a consensus GC-box (-83 to -74) and a proximal element (UPE, -54 to -38). The sequence of the UPE is highly related to the histone H4 subtype-specific element and it has been conserved in the duck H5 and the human and mouse H1° genes at equivalent positions. Although the effect of the UNE, GC-box and UPE was not tissue-specific, sequences from -38 to +77 appear to confer a degree of tissue specificity to the promoter. An activating erythroid-specific element (DE) was found downstream of the H5 gene (+1042 to +1185). The activity of the DE was modest but independent of position and orientation and required the presence of the promoter proximal elements. The DE harbors the sequence AGATAA that is recognized by a protein factor, presumably the same that binds to other erythrocyte-specific enhancers. The low activity of DE in the CFU-E may be related to the low concentration of the AGATAA-binding factor in the differentiation-blocked cells.

INTRODUCTION

Histone H5, a tissue-specific variant of histone H1, is an early marker of the avian erythroid lineage. Expression of H5 is regulated during erythrocyte differentiation so that its content increases with cell maturity (1). In the differentiation-blocked precursor cell line HD3 [CFU-(E) transformed by AEV (2, 3)], the H5 gene is transcribed, by and large, in a cell-cycle independent manner (1, 4). Although this is also true for the more mature and G1 arrested cells (1, 5), the rate of H5 gene transcription increases at the erythroblast stage and is maintained thereafter (1). This suggests that the H5 gene responds to lineage specific and stage-specific factors through regulatory sequences different from those of the replication-dependent histone genes.

We have analyzed the regulatory regions of the H5 gene by DNA-mediated gene transfer (6). The regions examined encompass all the DNase I hypersensitive sites previously mapped in the active chromatin (7), as well as those

coding for the protein. As recipient cells, we have used erythroid (HD3) and non-erythroid cells (fibroblasts). In a first analysis, we have used differentiation-blocked HD3 cells because their levels of H5 expression, although significant, are lower than those of more mature cells. This has permitted to determine both positive and negative promoter elements that control the basal transcription of the H5 gene. One of these elements (UPE) maps close to the TATA-box in a region of high homology among the genes coding poly(A)$^+$ mRNAs for replacement histone H1 subtypes.

While this work was in progress, two surveys of the regulatory regions of the H5 gene have appeared. Using microinjection in Xenopus oocytes, Wigley and Wells (8) have suggested that the region -85 to +313 (according to the +1 cap site determined by these authors) contains an enhancer-like element responsive to erythroid trans-acting factors. On the other hand, Engel and co-workers (9) have proposed the existence of a downstream enhancer element, the activity of which appears to be tissue- and stage-specific and independent of the promoter proximal DNA sequences. Our results indicate that the 3' flanking region of the H5 gene contains an element capable of increasing the activity of the promoter in non-induced HD3 cells. However, the activity of this enhancer is dependent on the presence of the promoter elements. The relative concentration of the presumed enhancer activating factor appears to be higher in immature adult erythrocytes than in HD3 cells.

MATERIALS AND METHODS

Construction of recombinant plasmids.

The p(5') plasmid series [p(5')3500 to p(5')38] contains the indicated length of DNA sequence upstream of the H5 gene, all ending at the AluI site (+77) in the H5 mRNA leader (initiation of transcription = +1, 10). These fragments were fused upstream of the chloramphenicol acetyltransferase reporter gene (CAT-SV40, 11) to generate H5-CAT-SV40 hybrid genes. The H5-CAT-SV40 genes were inserted at the EcoRV site of pAT153 (12) with the SV40 sequences closer to the EcoRI site of the vector.

5' promoter proximal deletions from the unique NheI site of pAT153 in p(5')386 were obtained by digestion with Bal31 and repair with T4 DNA polymerase in the presence of the four dNTPs. After digestion with ClaI, the relevant fragments were inserted between the EcoRV and ClaI sites of pAT153.

Internal deletions [pΔ series] from the SstII site (-38) were constructed by cloning a 1 kb SstII fragment of lambda DNA at the SstII site of p(5')90. A clone containing the MluI site of lambda, unique in the construct, nearest to the promoter distal sequences, was cleaved with MluI and digested with Bal31. After cleavage with SstII to remove the rest of the insert, the plasmids were repaired with T4 DNA polymerase, purified by gel electrophoresis and re-circularized.

Plasmid p(5')3500(3')3450 and deletants of it, was derived from a plasmid carrying H5 gene sequences -3500 to +3450 (EcoRI [linker site] -Asp718) by replacing the H5 mRNA sequences between +77 (AluI) and +835 (XmnI, in the H5 mRNA trailer, 37 bp upstream of the poly(A) addition site) by the coding sequences of the CAT gene [the 786 bp HindIII-Sau3A fragment from pRSVCAT (11)]. 3' deletions were constructed by excising the H5 downstream sequences, using an artificial XhoI site at the junction of the CAT-H5 sequences, and replacing them by the desired fragments. The hybrid genes were inserted at the EcoRV site of pAT153.

Plasmids pET90(5')ABS, and pET90(5')ABAS contained the filled-in AluI-BamHI fragment (+803 to +1365) inserted at the upstream NheI site of p(5')90 in the sense (S) or anti-sense (AS) orientation. Plasmids pET90(3')ABS and pET90(3')ABAS contained the AluI-BamHI fragment at the downstream ClaI site of p(5')90.

Plasmids pNTKCATS(386-95) and pNTKCATAS(95-386) were constructed by insertion of the SstI-NspI fragment of the H5 gene (-386 to -95) at the upstream BamHI site of ptkCAT, after filling-in and blunt-end ligation. Plasmids pNTKCATS(386-125) and pNTKCATAS(125-386) were created by inserting the corresponding SstI-AvaII repaired fragment upstream of the repaired BamHI site of ptkCAT. Plasmid pNTKCATS(115-95) was obtained by Bal31 deletion of pNTKCATS(386-95), and plasmid pNTKCATAS(371-386) was obtained by Bal31 deletion of pNTKCATAS(95-386).

All constructs were confirmed by restriction analysis and DNA sequencing (13, 14) of the junctions.

Cell growth and DNA transfection

HD3 (clone 41/2) (2) and H32 (15) were grown as described (7), except that the serum content of the medium was reduced by one-half. Primary chicken embryo fibroblasts (CEF) were isolated from 10-day old embryos as described (16), and grown as the H32 cells.

H32 or CEF cells (seeded at 0.8-1.5 x 10^6 cells/75 cm^2 dish the day before transfection) were transfected with 10 to 20 µg of plasmid DNA (linear range of response: 1-50 µg DNA) by the calcium phosphate method (6). HD3 cells (2x10^7, grown in suspension) were treated similarly except that they were subjected to a glycerol shock 4 h after transfection (17). Equal number of molecules of a given deletion series were used in the transfections. 2 µg of pRSVlacZ, coding for bacterial ß-galactosidase, were co-transfected with each hybrid gene for normalization purposes. The activity of the H5 constructs was not affected by the co-transfected pRSVlacZ in the proportions used in our studies. CAT (11) and ß-galactosidase (18) activities were assayed in cellular extracts (150 µl) prepared 40-48 h post-transfection, and the percentage of acetylated ^{14}C-chloramphenicol was determined by scintillation counting. The ß-galactosidase activities within a given series of transfections varied between 1.5- to 3-fold. The relative

CAT activities given in the text were corrected for by the efficiency of transfection (ß-galactosidase activity). p(5')90, and p(5')38 were included in each series of transfections to unambigously relate the effect of the deletions to promoter activity. Similar results were obtained when DEAE-Dextran was used for transfection (17).

S1 nuclease assays

A 350 bp filled-in BstNI-PvuII fragment of p(5')386, extending from -95 (H5 gene) to +250 (CAT gene), was subcloned at the SmaI site of M13mp19. The labeled single-stranded probe was synthesized as previously described (5). RNA (85 to 100 µg) extracted (19) from transfected or non-transfected HD3 cells was hybridized (20 µl) to 4 x 10^5 cpm of the probe in 70 % formamide, 0.4 M NaCl, 1 mM EDTA, 0.04 M Pipes (pH 6.9) at 56 °C. After 1 h, the mix was allowed to slowly cool down to 46 °C and was incubated 15 h at 46 °C. Annealed samples were chilled, diluted with 230 µl of 30 mM sodium acetate (pH 4.6), 1 mM $ZnCl_2$, 0.15 M NaCl, and digested with 50 U of S1 nuclease for 30 min at 37 °C. The products of the digestion were analyzed in 4 % polyacrylamide denaturing gels.

DNase I footprinting and interference assays

Nuclear proteins from HD3 and immature adult erythrocytes were extracted with 0.4 M KCl as previously described (20), except that non-ionic detergents were omitted during nuclei isolation.

0.5-2 ng of 5' end labeled DNA was pre-incubated at 25 °C with the nuclear extracts in the presence of 20 mM Hepes-NaOH (pH 7.6), 0.1 M KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol. 0.01% Nonidet P-40, 10 mM ß-glycerophosphate, 100 µg/ml poly (dI.dC), and digested with the appropriate amounts of DNase I for 2 min at 25 °C. DNA was purified and analyzed by electrophoresis in denaturing polyacrylamide gels.

For binding interference assays, the DNA was modified with dimethyl sulfate (13) or diethylpyrocarbonate (21) prior to incubation with the nuclear extract. Free and protein-bound DNA were separated by gel electrophoresis. DNA was extracted from the gel slices, hydrolyzed in 1M piperidine at 90 °C for 30 min and analyzed by electrophoresis in denaturing polyacrylamide gels.

RESULTS

Fig. 1A shows the physical map of the chicken histone H5 locus (10), and the location of the erythroid-specific DNaseI hypersensitive sites of chromatin (7). Regions upstream and/or downstream of the H5 gene were fused to the bacterial chloramphenicol acetyltransferase (CAT) coding region, and the hybrid genes were transfected into H5 expressing (HD3) and non-expressing cells [transformed quail fibroblasts (H32), or primary chicken embryo fibroblasts (CEF)]. To correct for differences in transfection efficiency, pRSVlacZ was co-transfected with the H5-CAT hybrid genes, and the

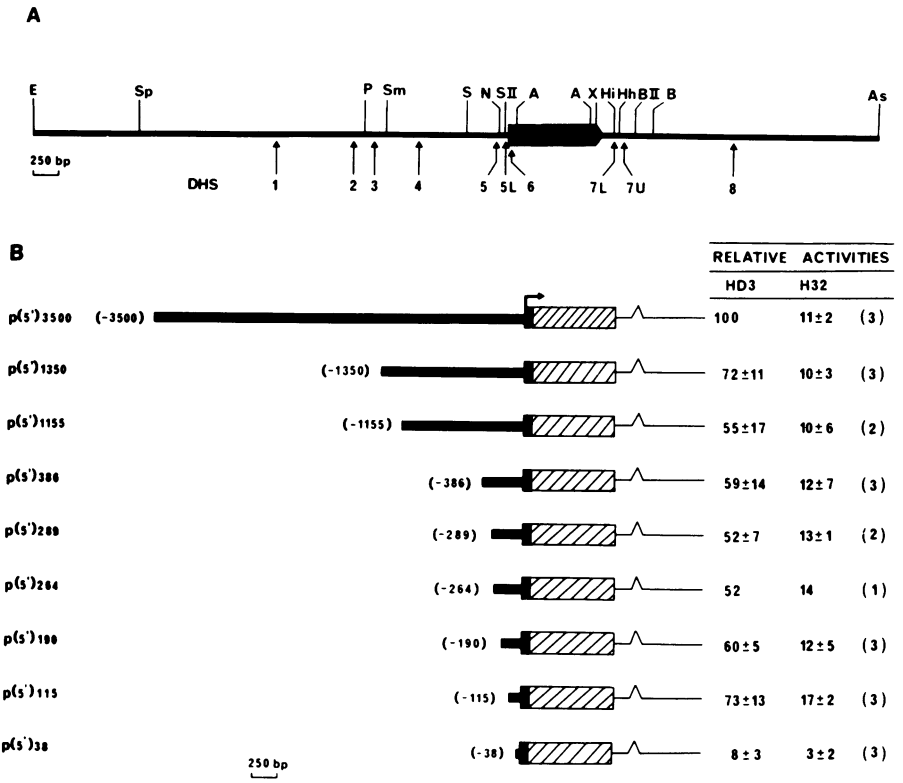

Fig. 1. Identification of cis-acting regions upstream of the histone H5 gene. (A) Physical map of the chicken H5 gene and flanking regions. The position of the DNase I hypersensitive sites (DHS) in chromatin is shown. The following restriction endonucleases were used in the construction of the expression recombinants: E (EcoRI, linker), P (PstI), Sm (SmaI), S (SstI), N (NspI), SII (SstII), A (AluI), X (XmnI), Hi (HinfI), Hh (HhaI), BII (BanII), B (BamHI), As (Asp718). The deletions in p(5')289 and p(5')264, p(5')190 and p(5')115 (see B) were produced by 5' Bal31 digestion of -386. (B) The 5' deletion mutants contained the indicated upstream sequences of the H5 gene (thick black line) fused at +77 to the CAT gene (cross-hatched box). The SV40 T-antigen splice/polyadenylation region is represented by the thin line. CAT expression of the hybrid genes is given relative to that of the longest recombinant in HD3. The values are the average of several independent experiments as indicated in brackets. HD3: erythroid cells; H32: transformed quail fibroblasts.

ß-galactosidase activity of the extracts was used to normalize that of CAT. Since similar CAT activities were obtained in H32 and CEF, only the results obtained with H32 are shown in the text.

Analysis of the sequences upstream of the H5 gene.

The longest construct contained H5 sequences extending up to -3500 and ending at +77, in the H5 mRNA leader [+1 corresponds to the mRNA start site previously described (10)]. Progressive removal of the promoter distal regions, including DHS-1 and DHS-2 in p(5')1350, and DHS-3 in p(5')1155 (Fig. 1B) resulted in a small, but reproducible, decrease in CAT activity in HD3 cells, whereas further deletions to -386, -289, -264, -225, -211, -190, and -115 had no significant effect (a selection of several deletion mutants is shown in Fig. 1B). The deleted sequences include DHS-4 at -841 (Fig. 1A), two S1 hypersensitive sites in supercoiled plasmid DNA at -366 and -283 (22), and an inverted consensus GC-box at -157 (TCCGCCCC). Further deletion of the -115 to -38 region severely impaired the activity of the promoter (Fig. 1B), indicating that the stronger transcriptional elements of the H5-CAT constructs reside in the promoter proximal sequences.

The deletion mutants consistently produced higher CAT levels in HD3 than in H32 (Fig. 1B), suggesting that the H5 promoter contains a tissue-specific element(s) or that our normalization standard (RSVlacZ) was preferentially expressed in H32. Results obtained with the HSV thymidine kinase promoter support the first alternative. Thus, the levels of CAT produced by ptkCAT, relative to ß-gal, in HD3 and H32 were the same (not shown). Since the activity of the tk promoter is mediated by ubiquitous transcriptional factors (23), this result suggests that the RSV promoter is also equally active in HD3 and H32 cells. The sequences that contribute to the erythroid-specific expression of the H5 promoter must, therefore, be located between -115 and +77, although a contribution of the distal region, -3500 to -1155, to this effect cannot be ruled out.

S1 protection analyses indicated that the transcripts were properly initiated at the H5 cap site. Total RNA extracted from transfected HD3 cells was hybridized to excess of a single-stranded probe that discriminates between the transcripts of the endogenous and transfected genes (Fig. 2A). The presence of a protected 249 nucleotide-long fragment (Fig. 2B) indicated correct transcription initiation of p(5')3500 and p(5')90. In the case of p(5')90, an additional transcript originating in the vector was also detected (346 nucleotide-long fragment, Fig. 2B). This transcript appears not to be translated into CAT since there is a direct relationship between the amount of the correctly initiated transcript and the CAT activity of the two constructs. In addition, the H5 promoter sequences upstream of CAT of the spurious transcript have stop codons in the three reading frames.

Promoter proximal elements

To analyze in greater detail the promoter proximal sequences from -115, a series of 5' deletion mutants were constructed with Bal31. These experiments (Fig. 3B) uncovered a transcriptional negative element (upstream negative element, UNE) located between -115 and -90 (Fig. 3A), the effect of

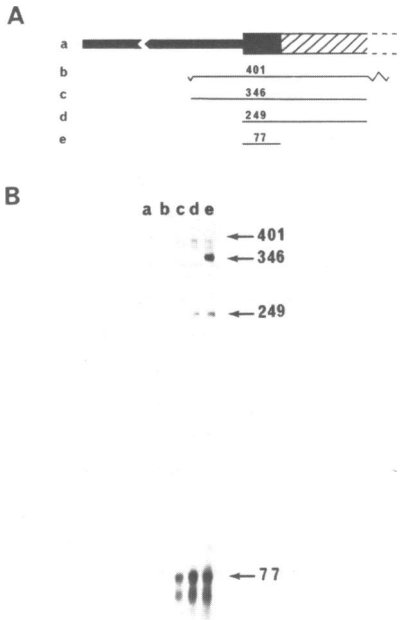

Fig. 2. S1 nuclease mapping of the mRNA start site. (A) The single stranded DNA probe (b) spans from the BstNI site (-95) of the H5 gene (thick black line) to the PvuII site (+250) of the CAT gene (cross-hatched), as shown in (a). The probe also contained primer and M13 sequences at the ends, as indicated by the zig-zags. mRNA started at the cap site of the hybrid and endogenous H5 genes should, respectively, protect 249 and 77 nucleotides of the probe (d, e), whereas RNA started upstream of the H5 promoter should protect up to 346 nucleotides (c). (B) Autoradiography showing the products of S1 digestion after hybridization of the probe with tRNA (lane b), RNA from mock-transfected HD3 (lane c), RNA from HD3 transfected with p(5')3500 (lane d), and RNA from HD3 transfected with p(5')90 (lane e). Non digested probe is shown in lane a.

which is not lineage-specific. The UNE encompasses DHS-5 previously mapped at the sequence [-115]TCCCTCCCT (7). An analysis of the UNE is presented below.

Further deletion to -81 reduced the strength of the H5 promoter by a factor of four [cf. p(5')90 and p(5')81, Fig. 3B]. This mutation removes part of a perfect consensus GC-box (24) (Fig. 3A) and its effect was not cell-specific. Nuclear extracts from chicken erythrocytes contain a DNA-binding protein that footprints on the GC-box (in preparation), suggesting that this element is recognized by a factor analogous to mammalian Sp1 (25).

Progressive deletions to -70, -59, and -54 had no significant effect on

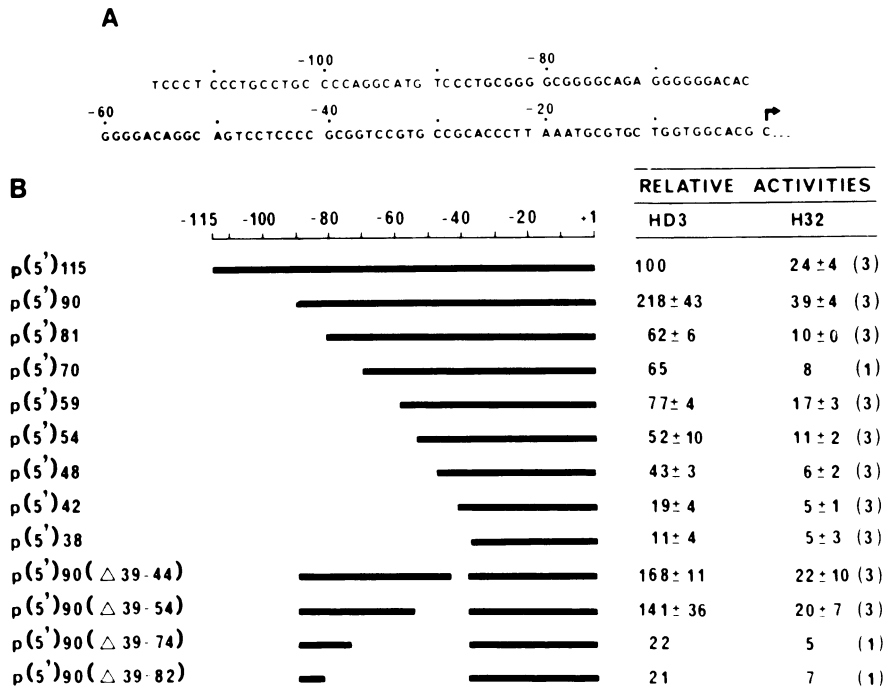

Fig. 3 Promoter proximal elements. (A) DNA sequence of the -115 to +1 region of the H5 gene. (B) Bal31 5' deletion mutants used in the analysis. p(5')90 and p(5')38 were produced by digestion with NspI and SstII, respectively (see Fig. 1A). The 3' internal deletions (Δ39-44) to (Δ 39-82) were created at the SstII site of p(5')90. Normalized average CAT activities of the hybrid genes are given relative to that of p(5')115 in HD3 cells. Figures given in parenthesis indicate the number of independent experiments.

the activity of the promoter. Deletions -54 to -42 defined the 5' boundary of a second upstream activating element (UPE) that includes sequences at least up to -38. Removal of those sequences results in a decrease in CAT activity in HD3 and H32 (Fig. 3B) suggesting that the trans-acting factor recognizing this element is ubiquitous. Internal Bal31 3' deletions from -38 confirmed the presence of two main activating elements, the GC-box and the UPE (Fig. 3B).

We have observed that the UPE is contained in a region of extensive homology between the promoters of the chicken H5 (10), duck H5 (26), mouse H1° (P. Lewis, personal communication), and human H1° (27). This region extends from -51 to -33 and is found 9 to 11 bp upstream of the TATA box (Fig. 8). The UPE appears to be a specific element of the sub-family of H1 genes encoding poly(A)$^+$ mRNAs for the replacement H1 variants. Interest-

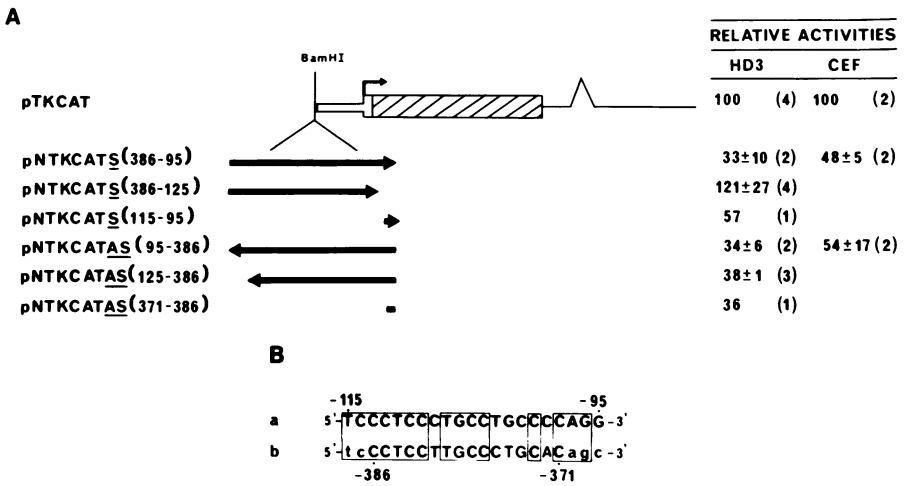

Fig. 4. Effect of the upstream negative element (UNE), and related sequences, on expression of the HSV tk promoter. (A) The region from -386 to -95 of the H5 gene, or the indicated deletions thereof, was introduced at the BamHI site upstream of the tkCAT gene [HSV tk (-109 to +51)] in the sense (S) or in the anti-sense (AS) orientations. The normalized average CAT activities of the recombinants are expressed relative to that of ptkCAT. Figures given in parenthesis indicate the number of independent experiments. CEF: primary chicken embryo fibroblasts. (B) (a) Sequence of UNE; (b) sequence of the reconstructed UNE-like element in pNTKCATAS(371-386) after fusion to tkCAT, given in the opposite orientation. Low case letters correspond to vector sequences.

ingly, the UPE sequence has strong homology to the H4 subtype-specific element recognized by H4TF2 (see Fig. 8 and Discussion).

The upstream negative element

To examine whether the effect of the UNE was specific for the H5 promoter, a -386 to -95 fragment, carrying UNE, was inserted upstream of ptkCAT and transfected into HD3 and CEF. Fig. 4A shows that the H5 sequences reduced the activity of the heterologous promoter when inserted either in the sense (S, [pNTKCATS(386-95)]) or in the anti-sense (AS, [pNTKCATAS(95-386)]) orientations. The magnitude and cell-specificity of the negative effect indicated that the activity of the UNE is independent of the type of promoter and cellular background. Moreover, deletion of the -124 to -95 sequences in pNTKCATS(386-125) resulted in an increase of CAT activity (Fig. 4A), confirming that the negative effect was due to the presence of the UNE. Progressive Bal31 deletions from -386 to -115 in pNTKCATS(386-95) further supported the conclusion that sequences upstream of -115 are not

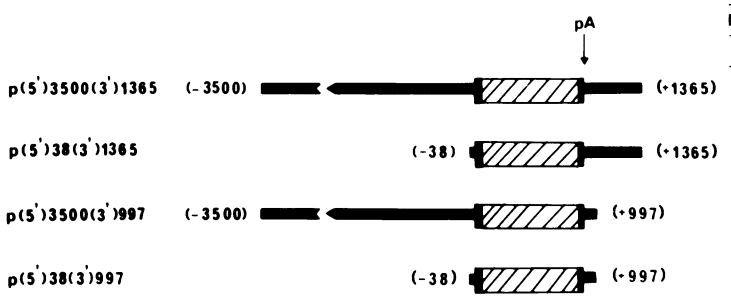

Fig. 5. Identification of cis-acting regions downstream of the H5 gene. Sequences from -3500 to +77 of the H5 gene [thick line] were fused to the CAT gene [cross-hatched] flanked by 3' sequences of the H5 gene [thick line] starting at the XmnI site (+851) of the mRNA trailer, upstream of the polyadenylation site (pA). Normalized CAT activities are expressed relative to that of the longest recombinant in HD3.

necessary for the negative transcriptional effect [only the result obtained with the shortest mutant, pNTKCATS(115-95), is shown in Fig. 4A].

The comparable CAT activities of pNTKCATS(386-95) and pNTKCATAS(95-386) suggested that the effect of the UNE might be independent of orientation and distance to the promoter. However, removal of the UNE sequences (-95 to -125) in pNTKCATAS(125-386) did not result in the anticipated transcriptional increase (Fig. 4A). Furthermore, progressive 5' to 3' deletions with Bal31 (not shown) indicated that the negative effect of the -125 to -386 fragment, from which the UNE had been deleted, was mediated by the -371 to -386 sequences (see pNTKCATAS(371-386), Fig. 4A). This paradox was resolved by inspection of the junction sequences of pNTKCATAS(371-386). This indicated that we had fortuitously reconstructed an inverted sequence highly homologous to the UNE, partly created by the fusion of the fragment to the vector sequences (Fig. 4B, a and b). Therefore, we conclude that the effect of the UNE can be mediated by similar, although not identical, sequences independent of their orientation. Comparison of the activities of pNTKCATAS(95-386) (containing the original and the reconstructed UNE) and pNTKCATAS(371-386) (containing only the reconstructed UNE) further indicates that UNE action is either not additive or sensitive to the distance to the proximal promoter elements.

Analysis of sequences downstream of the H5 gene

The effect of the 3' flanking regions on promoter activity was first examined using (5') H5-CAT-H5 (3') hybrid genes. In these constructs, the SV40-TAg sequences of the p(5') plasmids were substituted by the 3' sequences of the H5 gene, starting at the XmnI site (+851) of the H5 mRNA trailer, upstream of the polyadenylation signal (Fig. 1A, and ref. 28, 10).

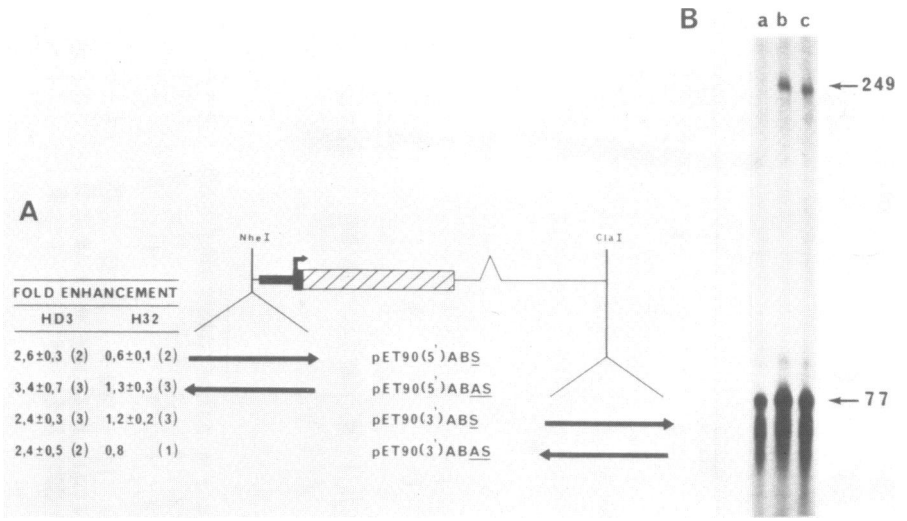

Fig. 6. The downstream activating element DE has enhancer-like properties. (A) The AluI-BamHI (+803 to +1365) fragment of the H5 gene was inserted in the sense (S) or anti-sense (AS) orientation upstream or downstream of p(5')90. Normalized average CAT activities of the recombinants containing the H5 downstream sequences is given relative to that of the parent construct. Figures in parenthesis indicate the number of independent experiments. (B) S1 nuclease protection of RNA from mock-transfected HD3 cells (lane 1) or HD3 cells transfected with pET90(5')ABS (lane 2) and pET90(5')ABAS (lane 3). The probe used was as in Fig. 2.

The reference construct, p(5')3500(3')1365 (Fig. 5), contains the region from +851 to +1365 of the H5 locus that includes the double DHS-7 (Fig. 1A, ref. 7). As it will be shown elsewhere, these sequences are sufficient for the processing of the primary transcript (M. Affolter and A.R.-C., in preparation). Expression of the hybrid gene was consistently about 20-fold higher in HD3 than in H32, whether or not the sequences up to +3450 were also present (i.e. including the lineage non-specific DHS-8, Fig. 1A, not shown). The increase in erythroid-specific expression of H5-CAT-H5 gene compared to H5-CAT-SV40 (cf. Figs. 1B and 5) suggested the presence of lineage-specific positive element in the 3' sequences. Consistent with this, a +1365 to +997 deletion resulted in 4-fold lower CAT expression in HD3 while no such effect was observed in H32 (p(5')3500(3')997, Fig. 5). Therefore, the activating erythroid-specific element (DE) is located between +997 and +1365, in agreement with the findings of Trainor et al. (9). Mutant constructs containing intermediate deletions (+851 to +1185, and +851 to +1042, not shown) revealed that DE is present between +1042 and +1185, in the region where the

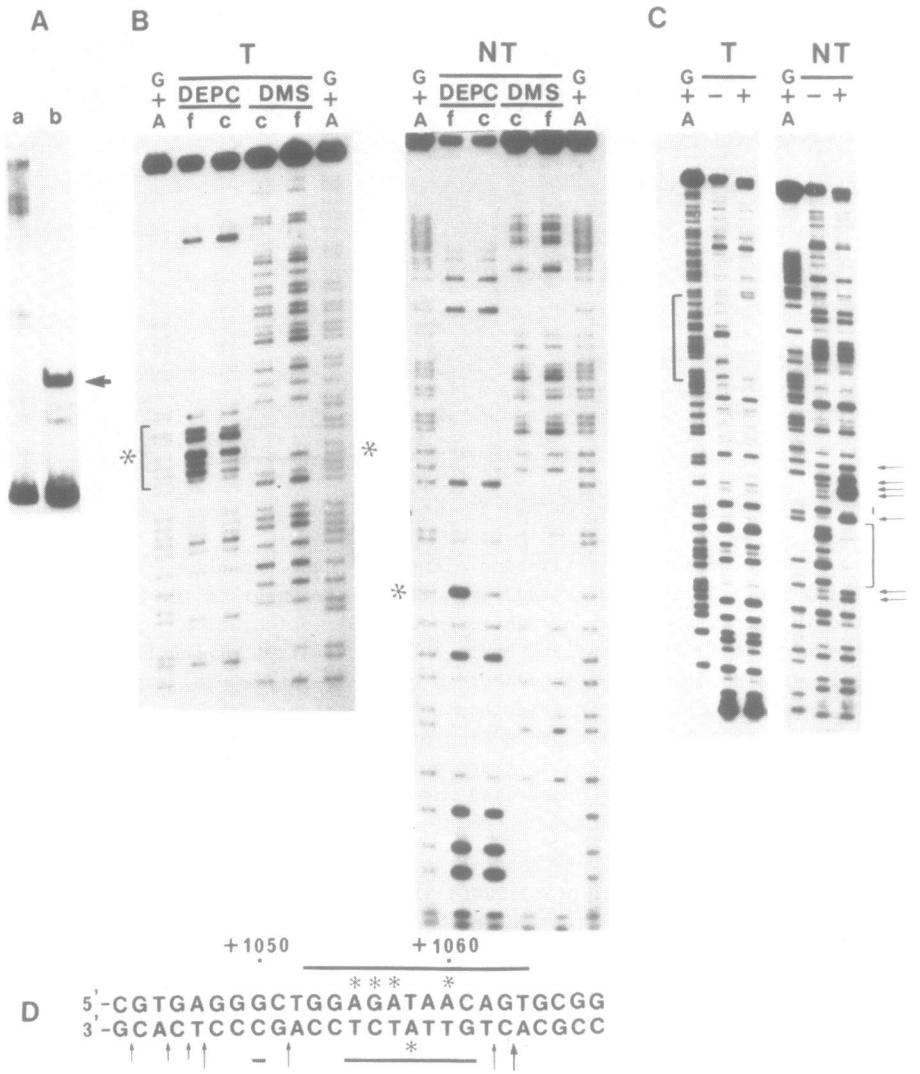

Fig. 7. Sequence recognition of the DE binding factor. (A) Gel retardation assay after incubation of a DNA fragment (+980 to +1087), containing the DHS-7 sequences of the DE, with equivalent amounts of HD3 (a), and immature adult erythrocyte (IE) (b) nuclear extracts. The arrow indicates the complex analyzed by modification interference in B. (B) Modification interference experiment. The DNA fragment, labeled at either 5' end, was partially carbetoxylated (DEPC) or methylated (DMS) prior to incubation with the IE nuclear extract. Free DNA (f), and the specific complex (c) are from an experiment similar to that in A. (C) DNaseI digestion of DNA incubated in the absence (-) or presence (+) of the IE nuclear extract. G+A, chemical

double DHS-7 was mapped (7) (see below). The action of DE requires the presence of the promoter elements since HD3 transfected with p(5')38(3')1365 only expressed background levels of CAT, as it was also the case for the double deletion in p(5')38(3')997 (Fig. 5). Therefore, DE cannot substitute for the positive promoter elements, as suggested by Trainor et al. (9), but it increases the strength of the promoter.

Because transcriptional and post-transcriptional effects of the 3' sequences cannot be easily distinguished in the above experiments, we have independently assessed the role of DE using enhancer-less constructs. Insertion of the +803 to +1365 fragment upstream or downstream of p(5')90 resulted in a 2.4 to 3.4-fold increase in CAT activity (Fig. 6A). This effect was specific for HD3 cells, and independent on the orientation and position. S1 protection analyses indicated that the mRNA produced by pET90(5')ABS and ABAS was initiated at the H5 cap site (Fig. 6B). The effect of DE requires the presence of the promoter activating sequences since insertion of the +851 to +1365 fragment downstream of p(5')38, in either orientation, resulted in no stimulation of transcription (not shown), in accordance with the results obtained with p(5')38(3')1365 (Fig. 5). Insertion, in either orientation, of the +803 to +1365 fragment upstream of the early SV40 promoter [$pA_{10}CAT_2$ (29), lacking the 72 bp enhancer] also resulted in an increase in CAT activity (not shown). However, the magnitude of the effect was somewhat lower than with the homologous promoter.

The relative small transcriptional effect of the DE is not due to the absence of other gene sequences, since inclusion of the coding sequences (+504 to +1365 or +4 to +851) in pET90(3')ABS failed to show a stimulation of CAT activity (not shown). Therefore, we examined whether the modest effect of DE was due to the limiting concentration of the DE trans-acting factor(s) in differentiation-blocked HD3 cells.

Gel retardation analyses using a fragment containing the region DHS-7 of DE (+980 to +1087) and nuclear extracts from immature adult erythrocytes indicated the presence of a complex (Fig. 7A) that was specifically competed by a synthetic oligonucleotide of the sequence +1050 to +1064 but not by oligonucleotides derived from the H5 promoter (not shown). Analysis of DNA in the complex purified by electrophoresis, in which the DNA was modified with diethylpyrocarbonate or dimethyl sulfate prior to incubation with the nuclear extract (binding interference assays) revealed that the binding factor recognized the sequence AGATAA of DE (Fig. 7, B and D). DNase I pro-

sequencing reaction. T and NT, template and non-template strand, respectively. (D) Summary of the results shown in B and C. (*) indicates the bases which modification interferes with factor binding. The DNase I resistant regions of the complex are shown above and below the corresponding strand. Arrows, DNase I hypersensitivity of the complex.

tection assays also demonstrated that the sequence AGATAA was recognized by a factor present in the immature erythrocyte extract (Fig. 7, C and D) but not in extracts from chicken lymphoid cells (results not shown). Since the relative concentration of the AGATAA-binding activity was much lower in HD3 than in the immature erythrocytes (Fig. 7A), we speculate that the weak activity of DE in undifferentiated HD3 reflects, among other factors, the limiting availability of this trans-activating protein.

DISCUSSION

Our analysis has located a negative (UNE), and two positive (GC-box and UPE) elements in the -115 to -38 region of the H5 promoter that are functional both in HD3 and fibroblasts. Although this suggests that the trans-acting factors are ubiquitous, one of them likely being the chicken homologue of mammalian Sp1 (25), the promoter constructs were consistently more active in the erythroid cells. This specificity must reside, at least in part, in the sequences between -38 and +77. In this respect, the H5 gene appears to be like the globin genes that also have tissue-specific promoter elements (30, 31). The tissue-specificity of the promoter is increased in constructs containing DE. Trainor et al. (9) have described the presence of a tissue-specific enhancer downstream of the H5 gene, in the same region where we have mapped DE. Although we do not find that DE can activate the -38 H5 promoter (cf. Fig. 5 and ref. 9), our results support their general conclusion. We have delimited the active enhancer region and demonstrated the presence of a nuclear factor that binds to the sequence AGATAA. This suggests that the enhancer activating factor is homologous to Ery f1 (32), EF1 (33), B2 (34), NF-E1 (35), and GF1 (36) that recognizes other erythrocyte specific genes such as the globins and the porphobilinogen deaminase (37). Sequences between -3500 and -115 had little transcriptional effect. This was unexpected since these regions harbor several lineage-specific DNase I hypersensitive sites (7). It is possible that the factors that recognize these sequences are either in limiting concentrations in HD3 or that their role is not transcriptional. Further work will be necessary to determine their function.

The sequence of the UPE and its distance from the TATA-box was found to be conserved in the H1 genes coding for replacement variants (Fig. 8), whereas it is absent in the replication-dependent H1 genes of vertebrates. We have traced the UPE homology back to the H1δ gene of the sea urchin S. purpuratus. This gene also encodes a poly(A)$^+$ mRNA for a slightly shorter H1 variant (as is the case of H5 and H1°) that appears to be regulated differently than other H1 genes (38). However, in the case of echinoderms the UPE homology is also shared by the replication-dependent H1 genes (39), suggesting that the specialization of this element occurred later during evolution. There is a striking homology between the UPE and the animal H4 subtype-

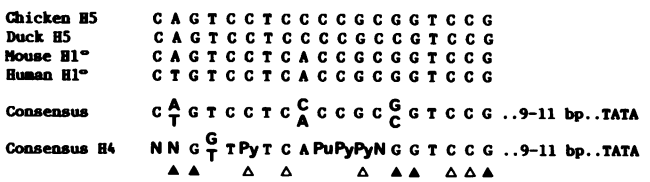

Fig. 8. Homology of the UPE to promoter sequences in H1 genes encoding poly(A)$^+$ mRNAs for replacement H1 variants and to the H4 gene subtype-specific element. The sequences of the duck H5 (26), mouse H1° (P. Lewis, personal communication), and human H1° (27) are aligned from their respective TATA-boxes. The H4 consensus was derived from the sequences of two chicken genes (47), three mouse H4 genes (48, 49), and two human genes (50, 51). The position of G sites in two different human H4 genes which are protected from methylation in vivo (51) or which methylation interferes with H4TF2 binding in vitro (41) are indicated by the arrowheads (in black those corresponding to the strand shown, and in white those corresponding to the complementary strand).

specific element required for H4 promoter activity (40, 41), also found at the same distance of the TATA-box (Fig. 8). The homology is nearly perfect at the H4 nucleotides which are in contact with H4TF2 (Fig. 8), a transcriptional factor that specifically recognizes the H4 element (41). We have recently observed that nuclear HD3 extracts contain a factor that binds to UPE sequences of the H5 and H4 genes (in preparation). It will be interesting to determine whether or not this factor corresponds to the chicken homologue of H4TF2.

The rate of H5 gene transcription is lower in HD3 than in IE (1). Our results suggest that the lower activity in HD3 may be effected, at least in part, by a suboptimal activity of the enhancer, likely related to the lower content/activity of the DE-activating factor in HD3 compared to IE. Although we were unable to induce differentiation of the HD3 subclone, an increase in H5 enhancer activity following HD3 differentiation has been observed by Trainor et al. (9) which is in keeping with the increase in relative concentration of the AGATAA-binding factor (33).

The H5 promoter is also down regulated in HD3 cells by the negative action of the UNE, a non-specific element which is also capable to reduce transcription of a heterologous promoter. The left part of the UNE is composed of a polypyrimidine tract while the right part is included in a perfect inverted repeat: TGCCTG(CCC)CAGGCA. Several silencers, including those of the ß-interferon (42), the apoCIII (43), the LDL receptor (44), and the lysozyme (45) genes also contain asymmetric polypurine/polypyrimidine tracts. We have previously mapped a DNase I hypersensitive site in the UNE region (DHS-5, Fig. 1A), and DNase I footprinting experiments have demon-

strated the presence of a nuclear factor that recognizes these sequences (in preparation). Since accumulation of H5 results in inhibition of DNA synthesis and arrest of the cells in G1 (46), the proliferating HD3 cells may need to keep the H5 gene under negative control to maintain the content of H5 at a level that does not interfere with growth.

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