Mutagenesis of conserved 5' elements and transcription of a chicken H1 histone gene

H.B. Younghusband*, R. Sturm and J.R.E. Wells

Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5000, Australia

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ABSTRACT
In addition to readily identifiable TATA and CAAT boxes, chicken H1 histone genes contain a highly conserved G-rich region at about minus 80 and an A-rich H1 histone gene-specific motif some 120 bases upstream from the H1 mRNA cap site. The level of transcription from wild-type, 'G-box' and 'A-box' H1 deletion mutant templates was tested in Xenopus oocytes and in HeLa cells. Removal of the H1 gene-specific motif had no effect on H1 gene transcription in either assay system, whereas deletion of the G-rich sequence decreased H1 mRNA levels by about ten-fold in oocytes and in HeLa cells. At least in heterologous systems, the H1-gene specific A-rich region does not appear to influence the level of H1 gene transcription.

INTRODUCTION
We have analysed regions upstream from histone H1 genes and have found several areas of DNA sequence homology (1). The so-called H1 box, 5' AAACACA 3', at about minus 120 is ubiquitous amongst all H1 genes reported and has an extended homology 5' AAGAAACACAAA 3' for chicken genes. This sequence is not present in the H1-related gene H5, which is only expressed in red cells (2). We have constructed deletion mutants of this A-rich motif and show that it does not influence transcription from H1 genes injected into Xenopus oocytes or transfected into HeLa cells.

A conserved G-rich sequence is also present in the promoter region of H1 genes (1). This motif (5' GGCGGG 3') is also found in the upstream region of histone variant genes, H5 (3) and H2A.F (Reference 4 and unpublished) as well as in a number of other polymerase II transcribed genes such as Herpes virus thymidine kinase (5) hypoxanthine phosphoribosyl transferase (6), SV40 (7) and adenosine deaminase (8). We show that this G-rich sequence substantially influences H1 gene expression in both Xenopus oocytes and HeLa cells.
MATERIALS AND METHODS

HI Gene Construct for Oocyte Transcription Studies

The 5 kb Bam HI fragment used for transcription studies contains an H1 gene and a divergently transcribed H2A/H2B gene pair cloned into the Bam HI site of M13mp8 (9). The parent construct is MCH5.0B (Fig. 1a) and derives from the recombinant λCH05 (10). The double stranded RF of each construct was used for microinjection.

Deletion Mutagenesis

M13 site-directed mutagenesis (11) was used to remove sequences corresponding to the H1 gene specific A-rich box and the G-rich region. The independent mutants are H1-AA and H1-AG (Fig. 1b). Synthetic oligodeoxynucleotides used for deletion procedures were 5' TGTGCTCGATTCTTTGGACTAA 3' and 5' CGTGCTAGCCGCCTGACGCGC 3'. After 5'-labelling, these oligonucleotides also served as probes to screen for potential mutant M13 phage (11), which were subsequently sequenced using an H1 gene-specific primer (9) to verify the precise structure of the mutants. In both mutants the deletion was as expected, removing 7 bases in H1-AA and 6 bases in H1-AG as shown in Fig. 1b.

Xenopus Oocyte Micro-injection

Procedures for oocyte handling and micro-injection were as described by Gurdon (12). For a given experiment, each of twenty-five oocytes was injected with 50 nl of solution containing 4 ng of DNA, the cells were incubated at 20°C for 18 hr and total RNA isolated (13).

HeLa Cell Transfection

The SV40 late replacement vector pJL4 (14) was linearized with Bam HI and the 5.0 kb fragment from MCH5.0B containing H1 and H2A/H2B genes was cloned into this unique site. Similar constructs were prepared from the H1-AA and H1-AG templates. In each case, recombinants for transfection studies were chosen such that the H1 gene was separated from the SV40 late promoter by the H2A/H2B gene pair. Thus, transcription from the H1 gene promoter is in the opposite direction from its adjacent H2A gene and from the SV40 late promoter.

About 2.5 x 10^6 HeLa cells per 10 cm plate were transfected with 5 μg of pJL4 vector plus insert DNA together with 20 μg sheared chicken DNA as carrier using the calcium phosphate technique (15). The media was replaced at 16-24 hr and the cells harvested 48 hr later. Cytoplasmic RNA was
prepared by washing the cells in PBS (.01 M phosphate, pH 7.0, 0.15 M NaCl),
detaching them by trypsinization, washing two more times in PBS and lysing
in 2 ml of 0.5% NP-40, 120 mM Tris pH 8, 10 mM NaCl, 10 mM EDTA for 15 min
at 0°C with occasional vigorous mixing. The supernatant from a clarifying
spin (2,000 xg, 10 min) was extracted with phenol/chloroform (1:1) and
nucleic acids precipitated with ethanol.

Quantitation of Transcript Levels

Specific primers, 5' labelled with $^{32}$P were used in excess to hybridize
to H1, H2A or H2B transcripts in total RNA isolated from oocytes or from
HeLa cells (9). The primers were extended on their templates using reverse
transcriptase and dNTPs; products were resolved on DNA sequencing gels and
visualized after appropriate exposure to x-ray film (5).

RESULTS AND DISCUSSION

The general approach has been to compare the level of H1 gene trans-
cription from recombinant plasmids containing the wild-type gene or the
deletion mutants. The DNA constructs also contain an H2A and an H2B histone
gene (Fig. 1) which served as internal controls. Transcription was measured
after DNA transfer in two systems. The first was a non-dividing system,
namely Xenopus oocytes. Although histone gene transcription is normally
associated with S-phase in dividing cells, the Xenopus oocyte has proved
valuable in studying histone gene expression (9,16,17). Dividing HeLa cells
were used in other experiments after DNA transfer by the calcium phosphate
technique. Because H2A and H2B are much more strongly expressed than H1
(especially in oocytes, see Fig. 2) exposures of X-ray film appropriate to
illustrate H1 transcription were often over-exposed for the other two gene
products. Nevertheless, shorter exposures of the same tracks allowed valid
comparisons to be made.

H1 Gene Expression and the A-Rich Motif

Four conserved sequences are found in the upstream region of H1 histone
genes (1). Two of these, the TATA and CAAT boxes are commonly found in the
promoter region of many polymerase II transcribed genes and these motifs
will not be discussed further here. The most 5' element 5' AAGAAACACAAA 3',
is specific for H1 genes whereas the G-rich element 5' GGGCGG 3' is found
upstream from a number of genes, particularly those with so-called house-
keeping functions (8).

The constructs MCH5.0B and H1-ΔA (Fig. 1b) were assayed for transcrip-
(a) HI

H1

H2A

H2B

-150

TGGTGCCAGAAATTCCGAGGAAAATACACTTTTGGTTAGTCCAAGA AATCAGGCACAACGAAG

-100

AATCAGGTTC TTAGCTGTG

-50

GGCTGCGGCGGTGCAGCG GGCGG GTTAAGCAACCCA CCAAT CACGGGCCTCTCTCTC TAAA

+1

GGCCGAGCTGCC OGGAATCGTTGCT

ATG TCC GAG ACC GCT CCC CCC

H1 primer

(b) H1-ΔA

deletion

5' AATCAGGTTTTATCTTGTAGCTGTG

3' AATCAGGTTTTATCTTGTAGCTGTG

5' AATCAGGTTTTATCTTGTAGCTGTG

3' AATCAGGTTTTATCTTGTAGCTGTG

H1 primer
FIG. 1: Histone Gene Insert for Transcription Studies

(a) Disposition of H1, H2A/H2B genes in a 5.0 kb fragment isolated from λ CHO5 (10). The insert was cloned into M13mp8 to form the construct MCH5.0B and the double stranded form of this template as well as deletion mutants derived from it were micro-injected into Xenopus oocytes for transcription studies. The 5.0 kb insert and the deletion mutants were also cloned into the SV40 vector pJL4 (14) for HeLa cell transfection experiments. Conserved regions, relative to the cap site at +1 are highlighted (see Ref. 1 for details). Oligodeoxynucleotide deletion primers used to delete the A-rich H1 box (H1-AA in the text) and the G-rich sequence (H1-AG in the text) are indicated. The position of the H1 primer used to quantitate transcription from the H1 gene is also shown.

(b) Deletion mutants of H1 histone upstream elements. DNA sequence analysis shows that precise 7 and 6 base-pair regions respectively were removed.

Transitional activity after injection into oocytes. The data in Fig. 2a show that deletion of the A-rich motif has no effect on the level of H1 gene expression in this system. It is common with primer extension analysis to observe
FIG. 2:

(a) Analysis of the H1-ΔA mutation on the level of H1 gene transcription in Xenopus oocytes. Track M contains pBR322 HpaII molecular weight markers. $^{32}$P-labelled 26 base-pair primers specific for chicken H1, H2A and H2B genes were extended on total cytoplasmic RNA from Xenopus oocyte which had been injected with the indicated H1 construct. The products were resolved on 6% polyacrylamide sequencing gels. The primers extend to 49 bases, 58 bases and 68 bases for H2A, H1 and H2B respectively and were visualized after exposure of the gel to x-ray film. H2A and H2B levels act as internal controls to compare H1 expression from the parent construct MCH5.0B (track 1) and the H1-ΔA template in which the H1-specific A-rich sequence has been removed (track 2).

(b) Expression from parental (track 1), H1-ΔA (track 2) and H1-ΔG (track 3) templates in the vector pJL4 transfected into HeLa cells. Again the H2A and H2B gene transcription levels act as internal controls to show the effect of each deletion on H1 gene expression. Total RNA from transfected HeLa cells was subject to primer extension analysis.

Several extension products (either from some heterogeneity of the 5' end of transcripts themselves or due to pause sites in reverse transcription of the template) and the less intensive of these for H2B and H2A are useful in showing that transcription from these internal control genes is comparable in each track.

In similar experiments with the vector pJL4 (see Materials and Methods) using calcium phosphate co-precipitation mediated DNA transfer to HeLa cells, again it is clear that expression of the H1 gene is not affected by
the H1-AA deletion (Fig. 2b). It is possible that the non-dividing oocyte lacks factors present in dividing cells which would interact with the H1 box and influence transcription, but the failure to see effects in HeLa cells does not support this proposition. Both systems are heterologous; nevertheless the A-rich motif is ubiquitous in H1 genes (including those from *Xenopus*, Ref. 1) and if it was associated with transcriptional control in a major way, effects should have been seen.

Conservation of a gene-specific motif such as the H1, A-rich sequence, over a wide evolutionary range (sea urchin, *Xenopus*, chicken, see Ref. 1) strongly suggests that it has a biological function. When it is aligned with a yeast *ars* consensus sequence the match is remarkable:

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Since *ars* activity has been correlated with S-phase expression of histone genes in yeast (19), it is possible that the H1 sequence is also associated with S-phase regulation of transcription of H1 genes.

**H1 Gene Expression and the G-Rich Motif**

In pioneering studies by McKnight and Kingsbury (5), linker-scanning mutations in the Herpesvirus thymidine kinase (tk) gene were analysed for effects on transcription in *Xenopus* oocytes. In addition to the TATA box mutations, two CG-rich upstream regions were identified as significant for the expression of this gene. Further studies showed these elements appear to be functionally analogous (20). The more distal C-rich sequence at about minus 100 (5' CCGCCC 3', see ref. 20) is in approximately the same relative upstream position as the H1-specific A-rich motif discussed above. Mutation of the C-rich region decreases tk transcription some 20-fold in contrast to the null effect of the A-rich region mutation in H1 genes. The more proximal region (at about minus 50) affecting tk gene transcription is a G-rich sequence, 5' GGCCGG 3' and is the inverted complement of the C-rich sequence (20). Mutations in this G-rich region also decrease tk transcription significantly. When the same sequence 5' GGCCGG 3' is deleted from the H1 gene (see Fig. 1b) a decrease in expression of about 10-fold is seen in the oocyte transcription system (Fig. 3). A similar result is seen for this H1-AG mutant template in
transient assays from transfected HeLa cells (Fig. 2, track 3). This result for H1 genes is not surprising given mutations in equivalent G-rich regions of the Herpesvirus tk gene and in the early promoter region of SV40 also result in decreased transcription (5,20-24). For SV40, it has been shown that the G-rich region binds a cellular transcription factor Spl (25) and this complex is necessary for optimal transcription (see Ref. 26 for review).

It has been proposed (23) that G-rich promoter motifs might facilitate recognition of a 'weak TATA box'. This might be particularly the case where multiple copies of the sequence exist such as in the 21 base pair repeats of SV40 (7) or in the promoter region of the adenosine deaminase gene (8).

Further studies will be required to establish the function of the H1-specific A-rich sequence. Given its similarity to ars sequences and its absence from the H5 gene, an H1 variant gene which is constitutively expressed through the cell cycle (27), it is possible that this sequence is associated with the initiation of H1 gene transcription in S-phase. The G-rich sequence upstream from H1 genes is shown here to modulate transcription (Fig. 2b and 3) and presumably does so via interaction with specific transcription factors.

We have isolated six chicken H1 genes and each codes for a variant protein as predicted by DNA sequence analysis. The way in which different levels of H1 sub-types are established in different tissues must result from
differential transcription, mRNA stability or protein turnover. The studies reported here represent initial investigations of transcriptional control mechanisms in HI gene expression.

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*Present address: Faculty of Medicine, Memorial University of Newfoundland, St.John's, Newfoundland A1B 3V6, Canada

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