Transcription of sea urchin histone genes in HeLa cells

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ABSTRACT

HeLa cells were transfected with recombinant DNAs containing the embryonic histone gene repeat of *Psammechinus miliaris* (h22) inserted in either orientation into a pBR-SV40 vector. After 2 to 3 days cytoplasmic RNA was analyzed for authentic sea urchin histone gene transcripts. The correct 5' termini of all five histone genes were detected, three (H2B, H2A and H3) at relatively high levels. In contrast, termination was largely aberrant with the correct 3' terminus of only the H2B mRNA found in significant amounts. The levels of histone gene transcription were dependent on the presence, but not the orientation, of SV40 DNA in the recombinant plasmid. The efficiency of initiation of transcription of the individual sea urchin histone genes in HeLa cells was very similar to that previously observed in Xenopus oocytes. The termination pattern, however, was quite different in that oocytes, all but the H3 gene terminate efficiently. The idiosyncrasies in termination efficiencies for these two heterologous transcription systems may reflect the presence of termination factors which are relatively species or even tissue specific and only some of which recognize the sea urchin "terminators" correctly.

INTRODUCTION

Transcriptional expression of the five histone genes from the common embryonic repeat of *Psammechinus miliaris* (h22) has probably been studied in more heterologous systems than any other eukaryotic genes. Results from these studies have shown a wide variation in the degree of transcriptional fidelity achieved. Not surprisingly in procaryotic and lower eukaryotic systems little specificity is observed. For example, in *E. coli* in vitro and in vivo studies of h22 subcloned into bacteriophage λ expression vectors showed that most transcription of the histone genes was under the control of λ promoters with some minor fraction of transcripts initiating from sites within the cloned h22 repeat (1). Similarly when the h22 repeat was inserted in yeast 2μm DNA, faithful transcription was not detected (T. Hohn and R. Grosschedl, unpublished). In *Xenopus laevis* oocytes, however, all five genes of h22 are capable of being transcribed with the correct 5' and 3'...
termini, but the efficiency of faithful initiation or termination varies strikingly from gene to gene. Correct initiation is particularly efficient for the H2A, H2B, and H3 genes while termination is efficient for all but the H3 gene (2). A very similar pattern of initiation of transcription is also observed in *Xenopus* blastulae injected as fertilized eggs with the histone repeat (3).

While it is difficult to fully rationalize this particular pattern of transcription, a plausible explanation is that initiation efficiency is primarily determined through the recognition or archetypal transcription signals (such as TATA boxes) while termination may be dependent on species or even tissue specific factors. This notion is supported by the recent demonstration of Stunnenberg and Birnstiel that the very inefficient termination of the H3 gene can be rescued by a specific sea urchin chromosomal protein isolated from 128-cell stage embryos (4). As a step toward investigating this theory further, we decided to analyze the mode of transcription of the h22 genes in another heterologous eukaryotic expression system. Thus human HeLa cells were transfected with the sea urchin histone gene repeat in order to ask whether the efficiency of initiation and particularly of termination differed significantly from that observed in *Xenopus*.

**MATERIALS AND METHODS**

The recombinant plasmids, pSR-h22 a and b, illustrated in Figure 1 were constructed as follows. An approximately 6.2kb EcoRI fragment containing one complete copy of the embryonic histone repeat of *P. miilarias* was excised from the plasmid pBR-2xh22, a pBR322 plasmid containing two tandem, head-to-tail copies of the histone repeat h22 (5). The histone-containing EcoRI fragment was inserted, in either orientation, into a pBR-SV40 vector (D. Picard and W. Schaffner, unpublished). In this vector the SV40 KpnI site near the 72bp repeated sequences was converted by means of synthetic linkers to an EcoRI site and the natural bacterial and viral EcoRI sites destroyed by fusing the SV40 EcoRI site to a pBR HaeII site.

For transfection experiments, recombinant plasmid DNAs in a circular, supercoiled form were purified by CsCl-EtBr centrifugation. HeLa cells were transfected (approximately 20 μg of DNA per 70% confluent 90mm plate of cells) via a modified calcium phosphate technique (6,7). Approximately 36 hours after transfection, the cells were trypsinized and reseeded onto plates with twice the surface area. Cytoplasmic RNA was harvested 2-3 days after
transfection. Plates of transfected cells were first washed with Tris-buffered saline (8) then scraped into 2.5ml/plate of cold lysis buffer (0.02M Tris-HCl, pH7.6, 0.05M KCl, 0.015M MgCl$_2$, 0.5% Triton-X-100). The gently lysed cells were centrifuged at 10,000g for 10 minutes at 4°C to pellet the nuclei and cellular debris. The supernatant was adjusted to 1% SDS, 0.01M EDTA, and 0.3M NaAc and the RNA purified by successive phenol chloroform extractions and then ethanol precipitated.

The S1 nuclease hybridization assays were performed essentially as described previously (2,9). Approximately 100 µg of RNA from transfected or mock-transfected HeLa cells was hybridized to $^{32}$P-labelled probe DNAs. In each experiment, a positive control was done with 5 µg of total RNA from P.miliaris embryos at the 128-cell stage of development. The DNA probes used in each experiment and the protected DNA fragments expected are illustrated in the figures.

RESULTS

Recombinant plasmids were constructed such that one complete copy of the sea urchin histone gene repeat (h22) was inserted into a pBR-SV40 vector (see Figure 1 and Materials and Methods). The vector contains pBR322
derived sequences, the entire SV40 early region, the SV40 origin of replication, the SV40 72bp repeated sequences, and a portion of the SV40 late region. The five histone genes were inserted, in either orientation, into a synthetic EcoRI site constructed at the former KpnI site of SV40.

The site lies approximately 50bp from the SV40 72bp repeated sequences, the "enhancer" sequences, which are known to activate early gene expression (10,11) and also to enhance the transcriptional activity of heterologous genes such as rabbit β-globin (7). HeLa cells were transfected with the recombinant plasmid DNAs via a modified calcium phosphate coprecipitation technique. After 2-3 days, cytoplasmic RNA was extracted from transfected and mock-transfected cells. The RNA was then analyzed by S1 nuclease hybridization assays for sea urchin histone gene transcripts with the same 5' and 3' termini as authentic sea urchin mRNAs.

The first set of S1 nuclease hybridization assays were carried out using a multi-probe technique capable of simultaneously detecting the correct 5' termini of all five sea urchin histone mRNAs (2). When authentic sea urchin RNA is hybridized to this $^{32}$P-5'-end-labelled probe and then S1 nuclease digested, five strong appropriately sized DNA fragments protected by the corresponding mRNAs are clearly visible (Figure 2, panel a, lane "Pm"). With RNA cells transfected with pSR-h22 a or b, bands corresponding to the correct 5' termini of H2B, H2A and H3 are seen. Faint bands of protected DNAs at the positions of H1 and H4 are also visible after long exposures indicating that the correct 5' termini of these genes are probably also made but in much lower amounts (Figure 2, panel a, lanes "a" and "b").

Two additional, relatively intense bands of approximately 485 and 250bp are present and these suggest that, besides faithful transcription starting at the normal initiation sites, discrete erroneous transcripts are also being made. In similar S1 nuclease hybridization assays examining the transcriptional expression of sea urchin histone genes in Xenopus oocytes, a prominent inappropriate band of protected DNA of approximately 250bp was also observed (2). Further evaluation of this band showed it to be due to an erroneous transcript which initiated after a pseudo-promoter sequence that lies within the H2B structural gene (C. Hentschel, unpublished data). Control experiments done in parallel with mock-transfected HeLa cells showed no visible bands of protected DNA fragments (Figure 2, lane "m"). Faint bands at positions 385 and 263bp are due to small amounts of renatured DNA probe fragments.
Figure 2. Multi-probe SI nuclease hybridization assays for the 5′-terminal sequences of h22 histone mRNAs. As illustrated in the diagram, the 5' multi-probe (32p-5′-end-labelled triple digest BamHI, BstNI and XhoI restriction fragments from 6.2kb HindIII h22 linear DNA) is predicted from previous work (2) to protect DNA fragments of the sizes indicated. Lanes "a" and "b" represent RNA from HeLa cells transfected with pSR-h22 a and pSR-h22 b, respectively. Lane "Pm" is with authentic embryonic RNA from P.miliaris and lane "m" is RNA from mock-infected cells. The DNA size marker is 32p-labelled HpaII fragments of a pBR322 recombinant plasmid (17).

In order to clearly establish that the strong bands seen after transfection with pSR-h22 a and b (Figure 2, lanes "a" and "b") truly represent DNA fragments protected by the 5' ends of H2B, H2A and H3 mRNAs, individual SI nuclease hybridization assays of the 5' terminus of each of these mRNAs were performed. The DNA fragments illustrated in the lower half of Figure 3 were isolated, 32p-5'-end-labelled, and then used as probes in three sets of SI nuclease hybridization assays. (Figure 3, panels a, b and c). In each case, RNA from HeLa cells transfected with pSR-h22 a or b (Figure 3, lanes "a" and "b") gave a protected DNA fragment that comigrated with the DNA fragment seen with authentic sea urchin RNA (Figure 3, lanes "Pm"). Thus, the results of these experiments using isolated DNA fragments as probes fully substantiate the results from the multi-probe 5' SI nuclease hybridization assays.

From these SI nuclease analyses we conclude that the efficiency of initiation of the five histone genes in HeLa cells is very similar to that previously found in Xenopus oocytes and blastulae. The next question was whether or not the sea urchin histone gene transcripts synthesized in HeLa
Figure 3. Single probe S1 nuclease hybridization assays for the 5'-terminal sequences of individual h22 mRNAs. Panels a, b and c depict S1 nuclease analyses of the 5' termini of H2B, H3 and H2A mRNAs, respectively. The \(^{32}\)P-5'-end-labelled fragments used as probes are drawn in the diagram with arrows indicating the protected DNA fragments expected. In each panel, lane "m" is a DNA size marker (see Figure 1), lanes "a" and "b" are with RNA from HeLa cells transfected with pSR-h22 a and b, respectively, lane "m" with RNA from mock-transfected HeLa cells, and lane "Pm" with authentic embryonic sea urchin RNA.

cells terminate at the normal sites. To answer this, RNA from transfected HeLa cells was analyzed by a similar multi-probe S1 nuclease hybridization assay but now using \(^{32}\)P-3'-end-labelled restriction fragments as probes (2). As illustrated in Figure 4, panel c, this multi-probe can detect the 3' terminal sequences of *P. miliaris* H2B, H3, H2A and H1 histone mRNAs. Figure 4, panel a, lane "Pm", illustrated this with authentic sea urchin RNA. RNA from HeLa cells transfected with pSR-h22 a or b gave numerous bands of protected DNA fragments, but only one appears to correspond 3' terminus, that of H2B (Figure 4, panel b, lanes "a" and "b").
Figure 4. SI nuclease hybridization assays for the 3'-terminal sequences of H22 histone mRNAs. Panel a is SI nuclease analysis with a 3' multi-probe (32P-3'-end-labelled HpaII/XhoI restriction fragments from 6.2kb HindIII H22 linear DNA) that is predicted from previous work (2) to protect the 3' termini of H2B, H3, H2A and H1 mRNAs (panel c). Panel b is SI nuclease analysis of the H2B mRNA 3' terminus using a single 32P-3'-end-labelled HpaII fragment as the probe (panel c). In panels a and b, lane "m" is a DNA size marker (see Figure 1), lanes "a" and "b" are with RNA from HeLa cells transfected with pSR-h22 a and b, respectively, lane "m" with RNA from mock-transfected HeLa cells, and lane "Pm" with authentic embryonic sea urchin RNA.

Parallel experiments with RNA from mock-infected cells showed no protection of the labelled DNA probe fragments (Figure 4, panel a, lane "m"). Thus, it appears that although a significant amount of transcription of the sea urchin histone genes is taking place in transfected HeLa cells, most of the transcripts terminate incorrectly. The possible exception is the H2B histone transcript. To substantiate this interpretation, SI nuclease analyses using a probe specific for the 3' terminus of H2B were carried out. With this probe, RNA from transfected HeLa cells gave a strong band of protected DNA that migrates with, or within a few nucleotides of, the band observed with sea urchin RNA (Figure 4, panel b). Thus within the resolution of this analysis, HeLa cells transfected with pSR-h22 a or b are synthesizing sea urchin H2B mRNA with the correct 3' terminus. Single probe SI nuclease analyses of the 3' termini of H3 and H2A mRNAs were also carried
out but, as anticipated, no bands corresponding to correct termination were observed (data not shown). Our results suggest that although HeLa cells transcribe the H2B, H3 and H2A sea urchin histone genes from the proper initiation sites, only the H2B RNA transcripts terminate properly.

DISCUSSION

In HeLa cells transfected with pBR-SV40-h22 recombinant DNAs, we have observed high levels of correct H2A, H2B, and H3 histone 5' termini and very low levels of H1 and H4 termini. This pattern is very similar to that observed after microinjection of histone genes into Xenopus oocytes (2) or fertilized eggs (3). Interestingly this pattern appears to be characteristic of the in vivo systems since in an in vitro system, HeLa whole cell extracts, only the H2A and H2B genes were transcribed with the correct 5' termini (12). This minor difference in transcriptional expression may arise because in the in vivo systems the newly introduced DNA is assembled into chromatin; whereas, in the in vitro system the DNA remains nucleosome free (13,14).

Irrespective of the transcriptional system, the most striking results from these comparative analyses is that three of the five histone genes (H2A, H2B, and H3) are potentially initiated at high levels while the remaining two genes (H1 and H4) are always poorly initiated. A possible explanation for the poor initiation of H1 and H4 genes is that they lack clear versions of the "TATA box" and the "CCAAT box" normally found in the promoter region of eukaryotic genes (15).

A major difference between the experimental design of the expression studies using oocyte microinjection and those described above using HeLa cell transfection is that in the latter case the histone genes were flanked by pBR-SV40 DNA sequences; whereas, in the former vector-free, recircularized histone gene repeats were used. The role of SV40 DNA sequences in influencing the expression of the histone genes is, as yet, not fully understood. Recent experiments, to be described elsewhere, suggest that SV40 influences the transcription of the sea urchin histone genes in both a replication-dependent and an enhancer-dependent manner (T. Gerster and W. Schaffner, manuscript in preparation). From our results, it appears that the role of SV40 is not orientation dependent. Similarly, in our constructions the distance between the SV40 72bp repeats and any given histone gene did not have a significant influence on its expression.

The most interesting observation from this study is that the efficiency of termination of the individual histone genes in HeLa cells is completely
different from that observed in *Xenopus* oocytes despite a striking similarity in transcription initiation. The mechanism of termination of these histone genes is dependent on the presence of a highly conserved hyphenated symmetrical sequence located downstream of the termination codons (16). While this sequence is essential, efficient termination also requires a considerable stretch of sequence downstream of the dyad symmetry. Furthermore, it seems probable that the function of the dyad symmetry element is more than that of promoting secondary structure in the RNA since it is the sequence *per se* and not just the symmetry which is evolutionarily conserved in histone genes (15). This suggests that a protein DNA interaction requiring this sequence is an integral part of the termination mechanism. The recent observation that the very inefficient termination of the sea urchin H3 gene in *Xenopus* oocytes can be rescued by a chromosomal protein from 128-cell stage sea urchin embryos (4) confirms this notion and also implies that such "termination factors" may be species or tissue specific. This observation together with our results suggests that the efficiency of correct termination in a heterologous transcription system may reflect partial and essentially coincidental recognition between the heterologous termination factors and the terminator sequences of the newly introduced genes.

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