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Chromatin structure determines accessibility of a hairpin polyamide-chlorambucil conjugate at histone H4 genes in pancreatic cancer cells

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Abstract

We have shown that a specific pyrrole-imidazole polyamide-DNA alkylator (chlorambucil) conjugate, 1R-Chl, alters the growth characteristics of various cancer cell lines in culture, and causes these cells to arrest in the G2/M stage of the cell cycle, without apparent cytotoxicity. This molecule has also shown efficacy in several mouse xenograft models, preventing tumor growth. Previous microarray studies have suggested that members of the histone H4 gene family, H4c and H4j/k, are the primary targets of this molecule, leading to reduced histone mRNA synthesis and growth arrest in cancer cells. In the present study, we examine the effects of 1R-Chl on transcription of other members of the H4 gene family, with the result that mRNA transcription of most genomic copies of H4 are down-regulated by 1R-Chl in a human pancreatic cancer cell line (MIA PaCa-2), but not in a cell line of non-cancerous origin (HEK293 cells). The basis for this differential effect is likely an open chromatin conformation within the H4 genes in cancer cells. Chromatin immunoprecipitation experiments show increased histone acetylation on the histone H4 genes in cancer cells, compared to HEK293 cells, explaining the differential activity of this molecule in cancer versus non-cancer cells.

Keywords

pyrrole-imidazole polyamide; chlorambucil; gene targeting agent; chromatin immunoprecipitation; MIA PaCa-2 cells

Small molecules or antibodies that target enzymes and receptors that are over expressed or mutated in malignant cells have reshaped modern cancer therapy [1–5]. However, DNA alkylating agents remain among the most common drugs for treatment of human cancer [6, 7]. Novel agents coupling DNA minor groove binders with alkylators have been introduced; however, due to limited DNA sequence selectivity of these agents, myelotoxicity is often the

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dose-limiting toxicity of these compounds [8–10]. New generations of sequence-selective DNA alkylating agents, based on the conjugation of established DNA alkylators such as chlorambucil (Chl) [11, 12] or CC-1065/CBI derivatives [13–16] to DNA selective pyrrole-imidazole (Py-Im) polyamides, have been developed with the aim of reducing such toxicity by limiting the number of DNA sites targeted in cells. Py-Im polyamides are a class of synthetic molecules that can be designed to recognize a wide repertoire of DNA sequences with remarkable affinity and selectivity [17, 18]. The polyamide-alkylator conjugates react covalently with DNA at the specific polyamide binding-site in the genome and inhibit transcription by stalling RNA polymerase during elongation [19].

Recently, an α-diaminobutyric acid-linked hairpin polyamide-Chl conjugate (1R-Chl, Fig. 1*a*) has been shown to bind and alkylate its cognate DNA sequence, both *in vitro* and in the cell nucleus, and to discriminate between match and single base mismatch sequences [12, 20, 21]. Previous studies have shown that 1R-Chl down regulates expression of histone H4 mRNA in various cancer cell lines, resulting in cell cycle arrest and a loss of cell proliferation and tumorigenicity in mouse xenograft models [20, 22]. The human genome encodes 14 genes coding for histone H4 protein, with each gene encoding exactly the same amino acid sequence, but with silent variations in DNA sequence. Microarray analysis pointed to the selective down-regulation of H4 family member H4c, and to some extent H4j/k expression, by 1R-Chl but not other H4 genes [20, 22]. By contrast, in the non-cancerous human embryonic kidney cell line 293 (HEK293), H4c transcript levels were not affected after treatment with 1R-Chl, and no cycle arrest was noted [20, 23]. Since each member of the H4 gene family harbors potential binding sites for 1R-Chl within their coding region (5'-WGGWGW-3', where W = A or T [12, 20]), it was unexpected that only H4c and H4j/k expression would be affected. Moreover, 1R-Chl treatment of human SW620 colon carcinoma cells lead to a reduction in total histone H4 protein levels [20]. These observations prompted us to examine whether 1R-Chl targets other members of the H4 gene family. We also explore the basis for the differential effect of this compound on cells of cancer versus non-cancer origin.

We chose to investigate the effect of 1R-Chl on mRNA levels for each member of the H4 gene family in both a cancer cell line and in a human cell line of non-cancer origin using quantitative RT-PCR. For our experiments, we chose the human pancreatic cancer cell line MIA PaCa-2, as previous studies showed that this cell line was highly sensitive to 1R-Chl treatment, in terms of down-regulation of H4c gene expression and blocking proliferation [23]. As a control cell line, we again chose HEK293 cells, where 1R-Chl was without effect on H4c gene expression or cell cycle arrest over the concentration range tested previously [23]. This cell line was immortalized by adenovirus-5 infection, but is not of cancer origin. We first sought to quantify the difference in sensitivity of these two cell lines to 1R-Chl treatment by monitoring cell growth using the MTS assay (Fig. 1*b*). By comparing the concentration of compound required for 50% reduction in cell number (~16 nM for MIA PaCa-2 cells, compared to ~400 nM for HEK293 cells), the data clearly show that MIA PaCa-2 cells are approximately 25-fold more sensitive to 1R-Chl than are HEK293 cells. One explanation for this difference could be the differential ability of the polyamide-Chl conjugate to penetrate these two cell types; however, previous studies have shown nuclear accessibility of Py-Im polyamides in this cell type [24] and nuclear accessibility of polyamide-chlorambucil conjugates in various cell types [15, 25]. We therefore examined the ability of a fluorescent version of the same polyamide, 1R-bodipy [20], to localize in the nucleus of HEK293 cells. Supplementary Figure 1*b* clearly shows nuclear staining of such cells after incubation in culture media for 16 h with 500 nM 1R-bodipy, thereby eliminating this trivial possible explanation.

We next determined the effect of 1R-Chl on histone gene expression in the two cell lines. We were able to generate primers for 11 of the 14 members of the histone H4 gene family. Family members “j” and “k” cannot be distinguished do to identical coding sequences, and we were unable to generate efficient primers for family members “f”, “g” and “i”. Treatment of MIA PaCa-2 cells with 1R-Chl at 50 nM led to down-regulation of all analyzed H4 mRNAs (Fig. 2*a*). In contrast, none of the H4 transcripts was reduced in HEK293 cells treated with 1R-Chl at the five-fold higher concentration of 250 nM (Fig. 2*b*). While previous microarray studies have identified H4c and H4k/j as the only H4 genes being down regulated by 1R-Chl in SW620 colon carcinoma and K562 chronic myelogenous leukemia cells [20, 22], we find that all H4 genes analyzed are affected by treatment with the polyamide conjugate in MIA PaCa-2 cells. To address this conundrum, we searched for H4 genes within the microarray data from Chou et al. [22] and found that the expression of many H4 genes was lower in 1R-Chl treated cells versus untreated cells, but only the data for H4c and H4j/k reached statistical significance (see www.ncbi.nlm.nih/geo/).

The differential effect of 1R-Chl in the two cell lines analyzed prompted us to hypothesize that the chromatin structure on the H4 genes in cancer cells may differ from that of non-cancerous cells, and that a more accessible chromatin in MIA PaCa-2 cells would explain the far greater sensitivity of this cell line to treatment with the polyamide. Chromatin immunoprecipitation (ChIP) assays were next used to determine the occupancy of histone H3 on the H4 genes, as a general measure for nucleosome occupancy. As shown in Figure 3*a*, on at least half of the H4 genes studied, the occupancy of histone H3 is lower in MIA PaCa-2 cells compared to HEK293 cells. Histone H3 occupancy on the β-actin gene was used as recovery standard to insure the reliability of the results. These findings suggest that some of the histone H4 genes may be depleted of nucleosomes within their coding regions in MIA PaCa-2 cells compared to HEK293 cells. We next asked whether a difference in histone acetylation might account for the difference in accessibility of the polyamide to the histone genes in these two cell types. In this experiment, we used antibody to acetylated histone H3 in ChIP assays (Fig. 3*b*), and plotted the ratio of acetylated H3 to total H3 in each sample to account for differences in total histone occupancy (Fig. 3*a*) and overall recovery. The data clearly show that histone H3 on the H4 genes in MIA PaCa-2 cells is highly acetylated compared to the same genes in HEK293 cells.

Our previous studies established that 1R-Chl blocks cancer cell proliferation through a two-hit mechanism, whereby down-regulation of histone gene expression leads to a general opening of the genome and subsequent widespread DNA alkylation, leading to a block in the G2/M phase of the cell cycle [23]. We provided both siRNA and chemical evidence that targeting members of the histone H4 gene family in cancer cells was the basis for the observed biological effects [22, 23]. Indeed, amongst a library of polyamide-Chl conjugates, only compounds that target the H4 genes were effective in down-regulation of mRNA levels and blocking cancer cell proliferation [22]. Comparison of the results from Affymetrix microarray studies in both SW620 colon cancer cells [20] and K562 chronic myelogenous leukemia cells [22], pointed to the H4c gene, and histone H4 protein, as the key target that elicits the observed growth effects of 1R-Chl. Down regulation of histone H4 or H3 protein with cognate siRNAs, followed by treatment with a control polyamide-Chl conjugate, which by itself was inactive, elicited similar growth inhibition as 1R-Chl [23]. Taken together, our data point to the H4 genes as the target of 1R-Chl responsible for growth inhibition, acting via the two-hit mechanism discussed above.

The current study sought to resolve two unanswered question raised in the previous work; namely, whether transcription of other members of the histone H4 family, besides H4c and H4j/k genes, are affected by 1R-Chl, and the molecular basis for the discrimination between cells of cancer and non-cancer origin by 1R-Chl. As reported previously [20, 23], 1R-Chl

shows a clear differential effect on MIA PaCa-2 pancreatic cells versus the human embryonic kidney cell line HEK293 (Fig. 1*b*). The present data provide a quantitative estimate of a ~25-fold greater sensitivity of MIA PaCa-2 cells to 1R-Chl, compared to HEK293 cells. We also show that mRNA levels of all members of the histone H4 gene family (for which qRT-PCR primers are available) are affected by 1R-Chl in MIA PaCa-2 cells, but not in HEK293 cells (Fig. 2), thus resolving the paradox that down-regulation of just a few members of the histone H4 gene family should result in a loss of total histone H4 protein, as mentioned above.

The differential sensitivity of cancer versus non-cancer cells to 1R-Chl prompted us to examine the chromatin composition of the H4 genes in these cells. We speculated that an open chromatin conformation on the H4 genes could account for the availability of polyamide binding sites in cancer cells versus cells of non-cancer origin, such as the HEK293 cells. Open chromatin could arise from the absence of nucleosomes or histone postsynthetic modifications that lead to more accessible DNA within the nucleosome. Indeed, ChIP experiments clearly show a reduction in histone H3 occupancy on some members of the H4 gene family in MIA PaCa-2 cells compared to HEK293 cells (Fig. 3*a*), suggesting lower nucleosome occupancy. But more striking, we find higher levels of histone acetylation on the H4 genes in MIA PaCa-2 cells compared to HEK293 cells (Fig. 3*b*). Histone acetylation is a well-established marker of open chromatin [26] and so it is reasonable to postulate that this modification and the subsequent change in nucleosome structure adopted through acetylation might well be the basis for the observed biological effects of 1R-Chl in cells of cancer versus non-cancer origin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Py-Im	pyrrole-imidazole
Chl	chlorambucil
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
ChIP	chromatin immunoprecipitation

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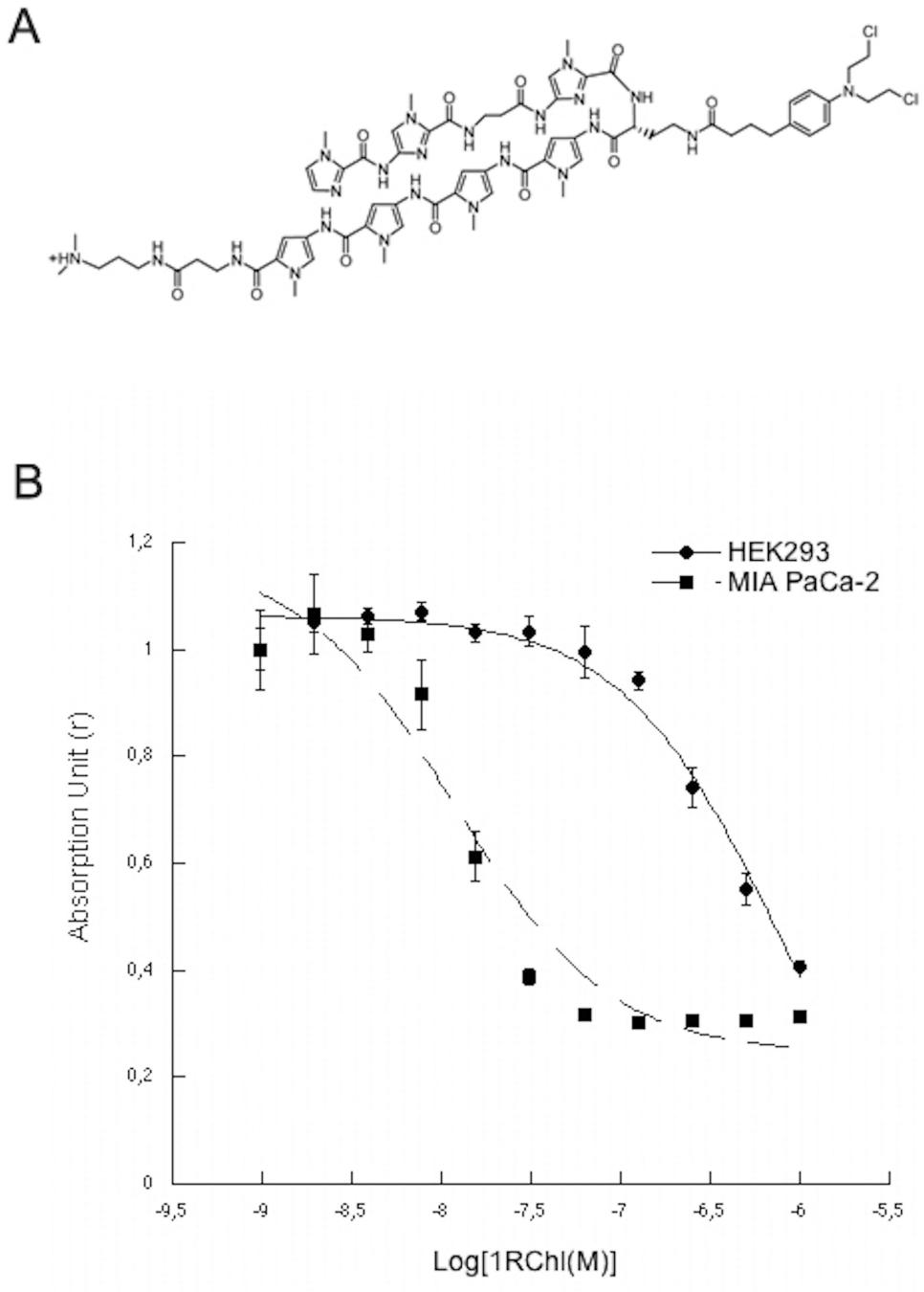

Figure 1.

(*a*) Polyamide-Chlorambucil Conjugate Structure. 1R-Chl = ImIm-β-Im-α(*R*-2,4-DABA^{Chl})-PyPyPyPy-β-Dp, where Py is pyrrole, Im is imidazole, β is β-alanine, Dp is dimethylaminopropylamine, and 2,4-DABA is (*R*)-2,4-diaminobutyric acid with α describing the amino acid linking the polyamide and Chl describing the chlorambucil substituent at the 4-amino position. (*b*) Comparison of the effect of 1R-Chl on the growth of MIA PaCa-2 and HEK293 cells. Cells were grown for 6 days in the presence of varying concentrations of 1R-Chl (from 1nm to 1μM) and cell viability at day 6 was measured with the MTS assay.

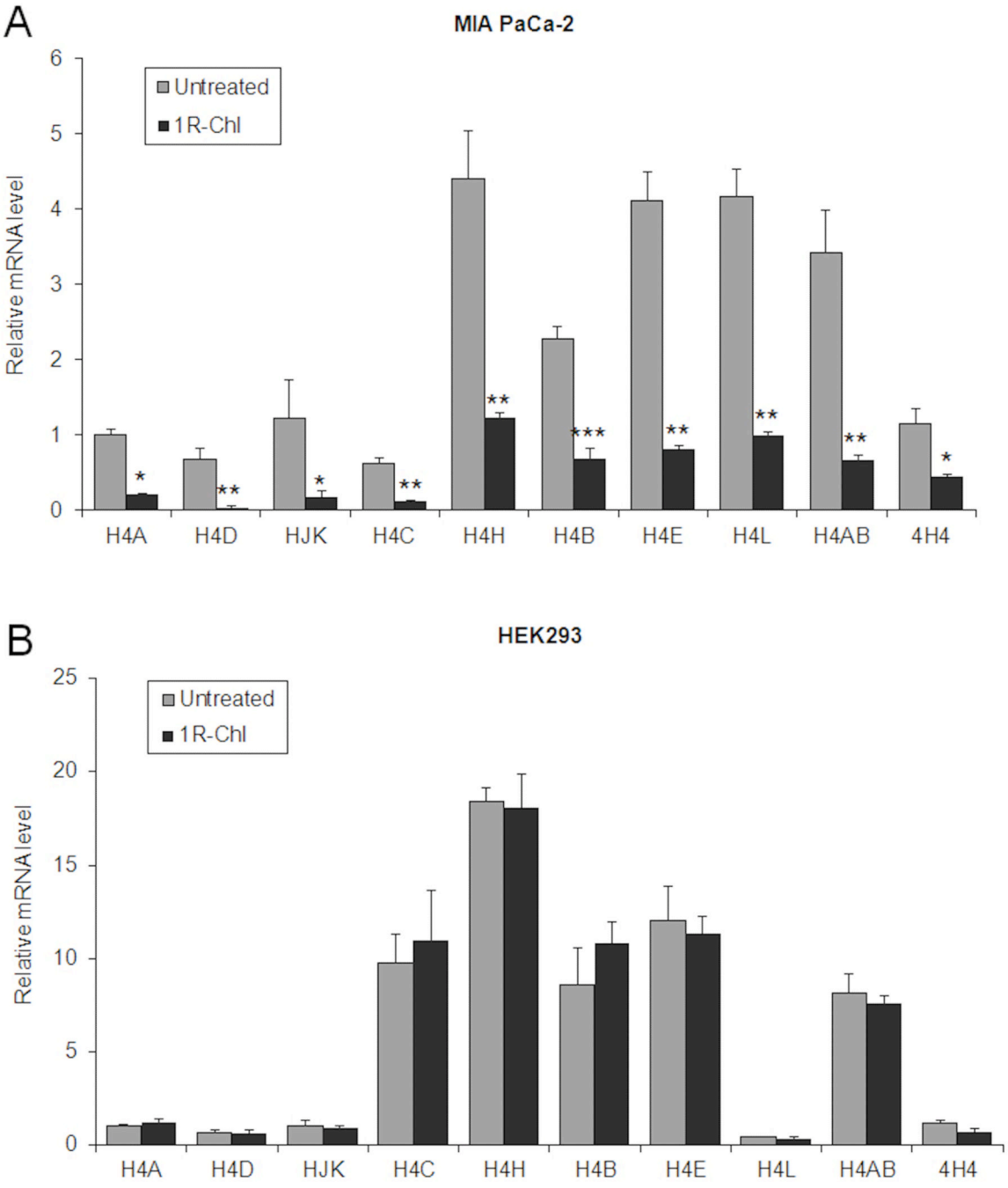

Figure 2.

H4 gene expression in MIA PaCa-2 and HEK293 cells. (*a*) MIA PaCa-2 cells were either untreated or treated with 50 nM 1R-Chl for 72 h. Shown are the relative mRNA levels of H4 genes (average and standard deviation of three independent experiments, each quantified in triplicate). GAPDH transcript level was used as recovery standard and the mRNA level of the H4A gene in untreated cells was set to 1. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. (*b*) HEK293 cells were treated as in (*a*) except for the concentration of the polyamide, which was 250 nM.

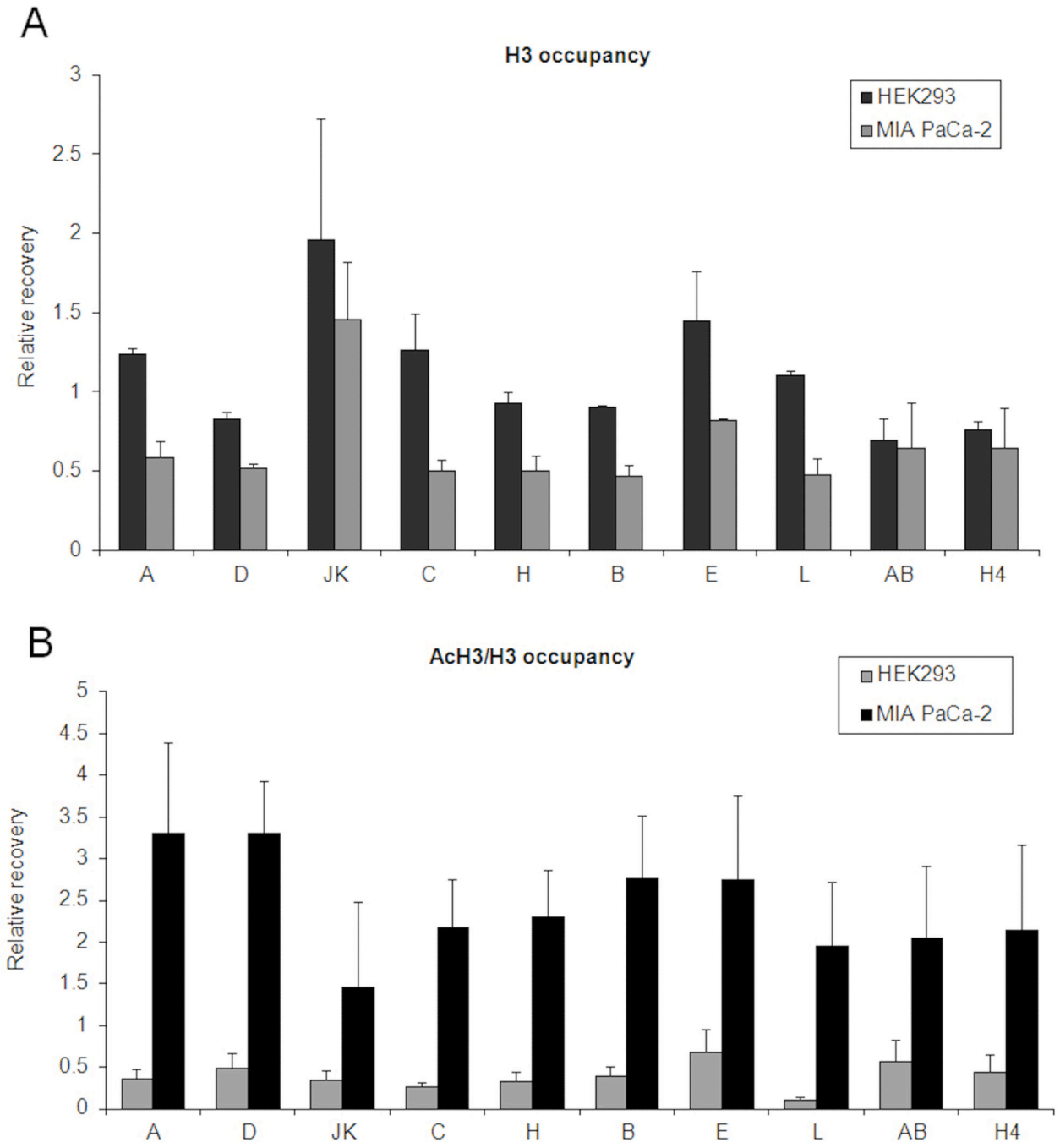

Figure 3.

(*a*) Histone H3 occupancy on the indicated H4 genes in MIA PaCa-2 cells and HEK293 cells. Chromatin immunoprecipitation was performed with anti-histone H3 antibodies. DNA recovery is expressed as relative to the recovery of β-actin coding sequence. Error bars are S.E.M. of two independent immunoprecipitations, each quantified in triplicate. (*b*) Ratio of acetylated histone H3 versus total histone H3 occupancy on the indicated H4 genes in MIA PaCa-2 cells and HEK293 cells. Chromatin immunoprecipitation was performed with anti-acetylated histone H3 and anti-histone H3 antibodies. Error bars are S.E.M. of two independent immunoprecipitations, each quantified in triplicate.