We have not determined the complete nucleotide sequence of this individual's other allele of the insulin receptor gene and do not know whether it is normal. The proband may be a compound heterozygote for two different mutations or, alternatively, the Val^{996} mutation may be dominant (*12*). Previous studies have shown the participation of acquired defects in insulin receptor tyrosine kinase activity in the pathogenesis of NIDDM (*13*). Furthermore, insulin-resistant patients have been described who may have primary genetic defects in their insulin receptor kinase activity (*14, 15*).

In conclusion, our data suggest that a defect in the insulin receptor tyrosine kinase can interfere with insulin action in vivo, thus supporting the hypothesis that mutations in the insulin receptor gene may be the cause of NIDDM, at least in a subpopulation of individuals (*12, 15–17*).

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Histone H5 in the Control of DNA Synthesis and Cell Proliferation

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The linker histones (H1, H5, H1°) are involved in the condensation of chromatin into the 30-nanometer fiber. This supranucleosome organization correlates with the resting state of chromatin, and it is therefore possible that the linker histones play an active role in the control of chromatin activity. The effect of H5 has been directly determined by expression of an inducible transfected H5 gene in rat sarcoma cells, which do not produce H5. Transfection resulted in the reversible inhibition of DNA replication and arrest of cells in G_1, at which time H5 concentrations approached that of terminally differentiated avian erythrocytes. The arrest of proliferation was accompanied by specific changes in gene expression probably related to the cell cycle block. The selectivity of these effects suggest that H5 plays an active role in the control of DNA replication and cell proliferation.

THE DIFFERENT LINKER HISTONE subtypes share similar structural features and bind, albeit with different affinity, to seemingly equivalent chromatin sites (*1*). The homologous variants H5 and H1° are synthesized in the maturing avian erythrocyte [H5 (*2, 3*)] or during the differentiation of mammalian cells [H1° (*4*)] and may have analogous functions. Expression of H5 and H1° is regulated independently of that of the main cellular histones [H1, H2A, H2B, H3, and H4] and is not coupled to DNA replication (*3, 5*). This results in increased amounts of H5 or H1° in terminally differentiated cells. H5, an early marker of the avian erythroid lineage, increases in relative content with the degree of cellular maturity as a direct consequence of an increase in transcription rate of the H5 gene and a progressive loss of the proliferation potential of the cells (*3*). The increase in linker histone content (H1 + H5) accompanies a progressive condensation of chromatin and the inactivation of most cellular genes (*2, 3*). Despite these correlations, the extent to which H5 contributes to the differentiation and loss of self-renewal capacity of the erythroid cells is still a matter of speculation.

We have directly determined the effect of H5 by transfection of a glucocorticoid-inducible H5 gene (pMSH5) (*6*) into rat sarcoma XC cells (*7*). This transformed cell line expresses relatively high levels of the glucocorticoid receptor (GR), and its growth characteristics are not noticeably affected by dexamethasone (Dex). In addition, XC cells do not differentiate, a trait important for distinguishing possible effects of H5 on cell proliferation from indirect effects due to cell differentiation.

The steroid hormone–responsive H5 gene and a selectable marker gene (*pRSVneo*) (*8*) were cotransfected (*9*) into XC cells, and G418-resistant colonies were isolated in which expression of H5 was hormone-dependent. An electrophoretic analysis of the basic nuclear proteins (*10*) from one such clone, XC10, grown in the presence and absence of 0.5 μM Dex, is shown in Fig. 1. The hormone induced the expression of a protein with the mobility of H5, not induced in the control XC8 cells, and its identity as H5 was confirmed by immunoblotting (*11*).

After hormone treatment, the amount of H5 in XC10 cells progressively increased and reached a maximum after 2 days (Fig. 1B) comparable to that found in mature erythrocytes (Fig. 1A) and, at the same time, the amounts of H1 decreased (Fig. 1B). The H5 content of XC10 cells began

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Table 1. Cell-cycle parameters of H5-expressing XC10 cells.

Days	Treatment*	% Cells in S + G_2 + M†	Time to double‡ (hours)	Rate of DNA synthesis ([^{3}H]Thy cpm per 10^5 cells)§
1		51	18 (±3)	455
1	Dex	29	45 (±3)	87
2		52	19 (±2)	492
2	Dex	23	62 (±4)	90
3		45	18 (±1)	560
3	Dex	24	52 (±3)	132
4		41	19 (±2)	610
4	Dex	31	40 (±5)	263

*Dexamethasone (0.5 μ*M*) was added to the culture at day 0. †Determined by flow cytometry (*3*). ‡Average. §Cells were pulsed for 30 min with 65 μCi/ml of [*methyl*-^{3}H]thymidine (80 Ci/mmol, DuPont Biotechnology Systems) at the end of each day in culture, and the radioactivity incorporated was measured (*3*).

to decrease after 2 days of Dex treatment because of inactivation of the MMTV promoter (see below), while there was a concomitant increase in the level of H1 (Fig. 1B). Continued incubation of XC10 in the presence of Dex for 2 weeks or longer resulted in further decrease of H5 and full restoration of H1. The apparent partial replacement of H1 by H5 most likely reflects a degree of competition between these histones for the same chromatin sites and the higher affinity of H5 for chromatin (*12*). However, the amount of H1 replaced was less than the input of H5 (Fig. 1B), as occurs during erythrocyte maturation (*2, 3*), an indication that the noninduced XC10 cells have less than one molecule of H1 per nucleosome or that H5 can also bind at sites other than where the linker DNA enters and leaves the nucleosome (*1*).

Comparison of several properties of H5 from XC10 cells and mature erythrocytes indicated that the histone is properly bound to XC10 chromatin. The H5 showed exclusive nuclear localization as determined from immunofluorescence with monoclonal antibodies to H5 (*11*) and was bound to nucleosome particles obtained by micrococcal nuclease digestion of nuclei (*13*). Moreover, H5 had a turnover rate similar to that of H1 and required, as expected from erythrocyte chromatin, a higher salt concentration than H1 for H5 to be extracted from nuclei (*13*).

The influence of H5 on the rate of proliferation was determined by comparing the growth of XC10 and XC8 cells in the presence or absence of 0.5 μ*M* Dex (*14*). Dex had no effect on the growth of the nontransfected XC8 cells (Fig. 2A), whereas it significantly reduced proliferation of H5-expressing XC10 cells (Fig. 2B). The variations in doubling time followed exactly the changes in H5 content of the XC10 cells (Figs. 1B and 2B). The doubling time increased to more than three times that of the controls in the first 2 days (Table 1), coincident with the maximum accumulation of H5 in chromatin (Fig. 1B), and slowly decreased at longer times (Table 1) as the content of H5 decreased (Figs. 1B and 2B). Analysis of the DNA content by flow cytometry (*3*) revealed that the number of cells in G_1 increased in parallel with the increase in H5 (Table 1). The percentage of cells traversing S and in G_2 + M was reduced by more than one-half after 2 days, whereas no such effect was seen with the control cells. The rate of [^{3}H]thymidine uptake into DNA in the H5-expressing cells was reduced to one-fifth of the control (Table 1), further indicating that the S-phase cells expressing H5 replicate DNA at a slower rate than those not expressing it. The growth arrest by H5 was not due to cytotoxic effects of H5 or the selection agent G418, since control and H5-expressing XC10 cells showed the same colony-forming capacity and since growth arrest also occurred in the absence of G418 (Fig. 2B). Moreover, the effect was not due to particular properties of the XC10 clone, since similar observations were made with other transfected cells (clones XC4 and XC20) also expressing high levels of H5 upon Dex induction.

The amount of H5 needed for the arrest of cell proliferation is similar to that found in mature erythroid cells arrested in terminal G_1 (Fig. 1A). High levels of H5 may be required if the histone has a preference in vivo to bind to chromatin organized in supranucleosome structures as it has in vitro (*15*). That is, H5 could cooperatively bind

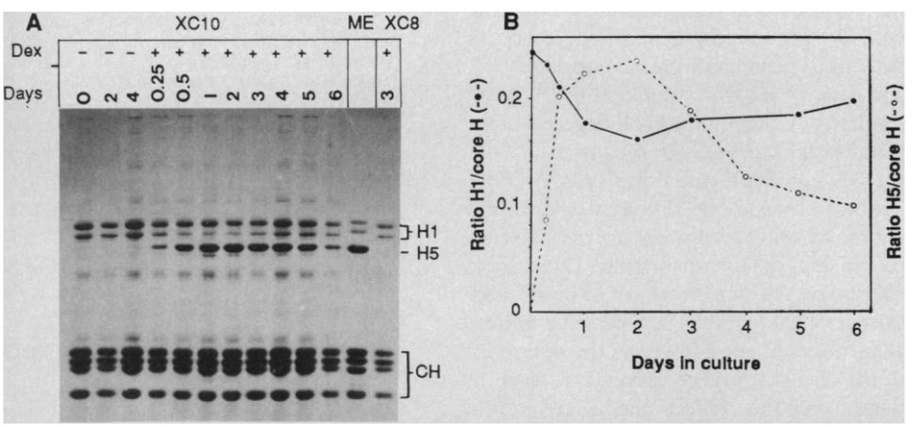

Fig. 1. Expression of chicken H5 in XC cells. (**A**) Basic nuclear proteins from XC10 cells transfected with pMSH5 (*6*), control XC8 cells, and mature chicken erythrocytes (ME), after separation by gel electrophoresis (*10*). The position of the linker histones (H1 and H5), core histones (CH: H3, H2B, H2A, and H4), and the number of days in culture in the presence (+) or absence (−) of Dex is indicated. (**B**) Variation of the H1 and H5 content, relative to that of the core histones, in H5-expressing XC10 cells. Ratios (±10%) were calculated by densitometry of duplicate gels and are averages.

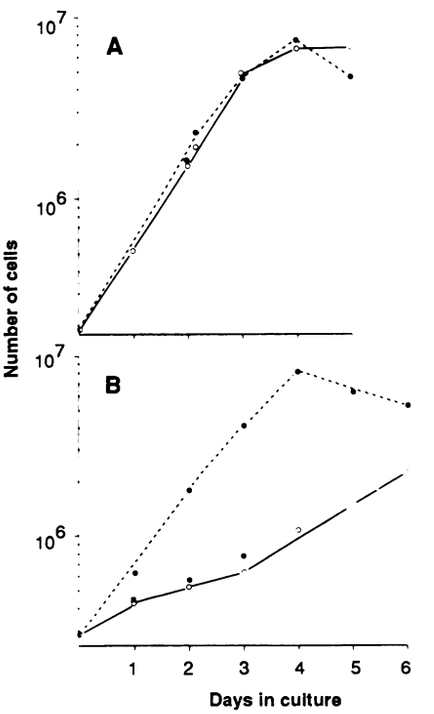

Fig. 2. Effect of H5 expression on cell proliferation. XC8 (**A**) and XC10 (**B**) cells were grown in the absence (●) or presence (○) of 0.5 μ*M* Dex, and the number of cells in each culture was determined at the indicated intervals (*14*). The growth curves in (A) and (B) are the average of two independent experiments. In a parallel experiment, Dex-treated XC10 cells were grown in the absence of G418 (■).

first to available sites in precondensed and condensed chromatin. Since condensed chromatin is normally inactive, H5 may not interfere with cell growth until lower affinity sites (for example, those in active chromatin) are filled. The effect of H5 would then be restricted to certain regions of the genome where it may render replication origins inaccessible and interfere with DNA elongation or inactivate genes required for entry into S phase, or do all three. Alternatively, H5 could randomly bind to chromatin and cause, in a dose-dependent manner, a nonselective inhibition of gene expression that would limit the amount of factors required for DNA replication.

In vitro experiments indicated that H5 had an effect in transcription. Incorporation of [^{3}H]UTP into RNA transcribed by RNA polymerase II or RNA polymerases I + III by nuclei of H5-expressing cells was about 50% that of the hormone-treated controls (*16*). This reduction could be due to a general decrease in the elongation rates of the RNA polymerases or to selective suppression of gene activity or to both. To distinguish between these effects, the steady-state levels of several mRNAs (*17*), with respect to ribosomal RNA (rRNA), were examined during the induction of H5 expression. After only 1 day there was a decrease in c-*myc* and the GR mRNA in H5-expressing XC10 cells (Fig. 3A), whereas no such trend was observed in the hormone-treated XC8 cells (Fig. 3B). The maximum decrease in mRNA and the subsequent recovery inversely correlated with H5 content (Figs. 1B and 3A). In contrast, the levels of the core histone mRNA examined (H3 and H4) increased parallel to the H5 content (Figs. 1B and 3A), while those of β-actin and vimentin were not significantly altered (Fig. 3, A and B).

The varied trends in the levels of the different mRNAs indicate that the effect of H5 is selective and that the changes in gene expression could be related to the cell cycle arrest. Thus, the increase in H3 and H4 mRNAs could be an indication that the H5-expressing cells proceed through early and mid G_1 before being arrested in late G_1, at a point after histone gene activation but before the onset of DNA replication. There is a precedent for such uncoupling in cell cycle mutants of baker's yeast (*18*). The alternative that the cells sense the influx of H5 as a signal to trigger histone gene activation is unlikely. Maximum levels of H3 and H4 mRNAs were attained after a substantial amount of H5 had been deposited in chromatin, and the subsequent decrease in core histone mRNA occurred while the amount of H5 was relatively high (Figs. 1B and 3A).

The decrease in the mRNA levels of c-*myc* and the GR mRNA is probably also related to cell cycle arrest. The c-*myc* proto-oncogene, coding for a cell proliferation competence factor, is down regulated in nonproliferating cells (*19*). Similarly, expression of the GR is lowest in the G_1 phase (*20*). The lack of effect of H5 on the levels of β-actin and vimentin mRNAs (Fig. 3) is also in keeping with arrest in G_1 since vimentin is preferentially expressed in G_1 (*21*), and the levels of β-actin are not particularly affected during the cell cycle.

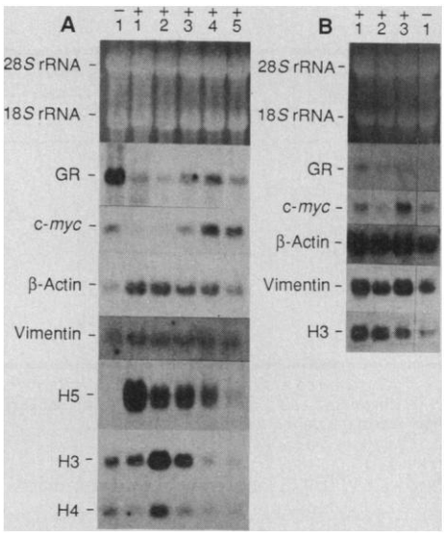

Fig. 3. Effect of H5 on gene expression. Total RNA from H5-expressing XC10 (**A**) and control XC8 cells (**B**) was separated by electrophoresis and blotted onto membranes (*17*). The same membranes were successively hybridized with the indicated probes. A compilation of the autoradiographs is shown. The days in culture in the presence (+) or absence (−) of 0.5 μ*M* Dex is indicated. Ribosomal RNA was visualized by ethidium bromide staining.

The decrease in the amount of H5 mRNA (Fig. 3A) is probably not relevant to the effect of H5, since it is known that the MMTV promoter becomes refractory to prolonged stimulation by the hormone (*22*). The lack of recovery of H5 mRNA could result from the binding of negative transcriptional factors (*22*) or the lower levels of the GR in the H5-containing cells, or both.

These observations indicate that there is a concerted cellular response to the presence of H5 but do not distinguish cause from effect. The cell cycle block could be a consequence of the effect of H5 on gene expression or vice versa. However, the selectivity of the changes in gene expression, some of which probably reflect alterations in the transcription rates of the corresponding genes (*23*), suggests that the mere presence of H5 is not sufficient to inactivate transcription. Previous observations have also indicated that the template capacity of genes specific to the erythroid lineage (for example, β^A-globin and histone H5 genes) is virtually the same in erythroblasts and mature erythrocytes (*3*) despite the higher linker histone content of the mature cells. Nucleosomes (*24*) or supranucleosome particles (*25*) harboring these active gene sequences contain H1 and H5 as does the inactive chromatin. Therefore, the presence of H5 appears to have no effect on the activity of at least differentiation-specific genes (erythrocytes) or genes expressed in G_1 or constitutively expressed during the cell cycle (XC cells). Unless the active effect of H5 is directly or indirectly restricted to growth-related genes, these observations suggest that the alterations in gene expression are the consequence rather than the cause of the cell cycle block.

Regardless of the mechanism of action, the active role played by H5 in the control of cell proliferation suggests a crucial role for the histone during the differentiation of the avian erythrocyte. Given the structural relatedness between H1° and H5, it is likely that H1° is instrumental in arresting the cell cycle during the differentiation of mammalian cells. It will be interesting to determine whether these effects are specific for the replacement linker histones or can be mimicked by any H1 subtype, provided that they are constitutively expressed, in sufficient amounts, during the cell cycle.

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Rapid β-Adrenergic Modulation of Cardiac Calcium Channel Currents by a Fast G Protein Pathway

ATSUKO YATANI AND ARTHUR M. BROWN

β-Adrenergic agonists activate the G protein, G_s, which stimulates cardiac calcium currents by both cytoplasmic, indirect and membrane-delimited, direct pathways. To test whether β-adrenergic agonists might use both pathways in the heart, isoproterenol was rapidly applied to cardiac myocytes, resulting in a biphasic increase in cardiac calcium channel currents that had time constants of 150 milliseconds and 36 seconds. β-Adrenergic antagonists of a G protein inhibitor blocked both the fast and slow responses, whereas the adenylyl cyclase activator forskolin produced only the slow response. The presence of a fast pathway in the heart can explain what the slow pathway cannot account for: the ability of cardiac sympathetic nerves to change heart rate within a single beat.

STIMULATION OF CARDIAC SYMPATHETIC nerves releases norepinephrine, which increases the rate and force of the heartbeat through activation of β-adrenergic receptors. β-Adrenergic agonists such as isoproterenol (ISO) imitate these effects and increase Ca^{2+} currents through a cytoplasmic, adenosine 3′,5′-monophosphate (cAMP) pathway (*1*) having latencies of 2 to 20 s and half-times of 10 to 100 s (*2*). The G protein G_s (*3*) is a branch in this signaling pathway and increases Ca^{2+} currents by both membrane-delimited, direct and cytoplasmic, indirect pathways (*4, 5*). By analogy with direct muscarinic activation of atrial K^+ channels, the direct G_s pathway could produce responses to ISO at least ten times faster than those observed (see Fig. 1C). However, in previous experiments, the concentration of ISO was not changed quickly enough to see a fast response. To test for the presence of a fast response, we recorded whole-cell Ca^{2+} channel currents from single cardiac myocytes (*6*) and applied rapid jumps of ISO with the concentration clamp method (*7*).

With this method, the response time was 10 ms (Fig. 1, A and B) and the changes in membrane currents observed at longer times reflect the traversal times of the pathways of interest. As a first step, we applied the muscarinic agonist carbachol (20 μM), which produced a K^+ current (Fig. 1C) with a delay of 35 ± 7 ms and a subsequent activation time constant (τ) of 650 ± 130 ms (n = 4), similar to values reported elsewhere (*8*). For Ca^{2+} currents, the situation is different because G_s—unlike the G protein G_k, which is obligatory for opening of atrial muscarinic K^+ channels—is only a modulator (*5*). To produce Ca^{2+} channel currents, depolarizing voltage-clamp steps were required, and to increase the amplitude of the currents Ba^{2+} was used as the charge carrier (I_{Ba}) (Fig. 1B). There is an additional complication in that after the whole-cell recording configuration has been established, the high threshold, dihydropyridine (DHP)–sensitive Ca^{2+} channel currents that we were studying initially increased in amplitude and then exhibited rundown (Fig. 1D) (*9*). Concentration steps of ISO were applied after the rundown had begun its slower phase.

In 20 experiments, ISO (0.1 nM to 10 μM) was applied 50 to 300 ms before the depolarizing test pulse (Fig. 2, A and B). In all cases, the first pulse after ISO treatment exhibited an increase in peak current amplitude and a slowing of inactivation (Fig. 2A). At concentrations above 10 nM the amplitude of the peak current in subsequent pulses continued to increase (Fig. $2A_3$) but the subsequent rate of increase was much slower, giving a biphasic appearance to the plot of the peak current versus time (Fig. 2B). Beginning with the second pulse after ISO (10 s later) the current waveform became similar to control so that the increased currents were simply scaled enlargements of the control waveform (Fig. 2B, insets 1 and 2). The peak current-voltage curve recorded in the steady state (at 60 s) was also unchanged from control. In each experiment, step changes of control solutions had no effects on current amplitude or waveform. In seven additional experiments with ISO at 2 μM, we fitted the slow increase with a single exponential function after a delay. The time constant was 36 ± 4 s, similar to other rates of increase of Ca^{2+} channel

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