Inhibition of Proliferation of Primary Avian Fibroblasts through Expression of Histone H5 Depends on the Degree of Phosphorylation of the Protein

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Abstract. To obtain stable and constitutive expression of histone H5 at levels comparable to those observed in normal chicken erythrocytes, an avian self-inactivating retroviral vector was used to transfer the H5 gene into cells which do not express this protein. The vector, pDAH5, was obtained by removing the CAAT and TATA boxes of the 3'LTR of the avian leukosis virus RAV-2 and inserting the H5 sequence.

Infection of QT6 quail cells with the recombinant virus (DAH5) led to the stable integration of the foreign H5 gene at low copy number, to the formation of correctly initiated mRNA transcripts and to the production of H5 protein. The amount of H5 expressed was equivalent to that of a mature chicken erythrocyte.

Expression of histone H5 in DAH5 transformed cells, such as QT6 or AEV-ES4, transformed chicken embryo fibroblasts had only slight effects on the growth rate and did not inhibit cell replication. Conversely, the effect of H5 expression on normal quail and chicken fibroblasts was dramatic: cells acquired the aspect of quiescent fibroblasts, grew very slowly, and nuclei looked compacted, often extruded from the cell. The H5 histone produced in QT6-transformed cells was found to be phosphorylated while in normal chicken fibroblasts the protein lacked this posttranslational modification. It is proposed that the chromatin-condensing role of histone H5 is inhibited by its phosphorylation.

H5 is a variant of the H1 family and represents an extreme example of tissue specificity, being found only in nucleated erythrocytes. It binds tightly to chromatin and its association with DNA induces pronounced modifications of DNA conformation. H5 shows also strong preference for higher-order structure of chromatin (Mura and Stollar, 1984; for review, Wu et al., 1986). Such preferences might be the basis of the higher stability of H5-containing chromatin.

In avian erythropoiesis, the process of cell maturation is accompanied by a progressive condensation of the chromatin leading to a shutdown of DNA replication and concomitantly, the accumulation of H5. Recent studies using microinjection of H5 in L6 rat myeloblasts (Bergman et al., 1988) or transient expression of H5 sequence into rat sarcoma cells (Sun et al., 1989, 1990) have shown that overexpression of H5 inhibits replication. Metabolic studies have indicated that H5 is phosphorylated immediately after its synthesis and subsequently becomes dephosphorylated as the erythrocyte matures (Sung et al., 1977). Significant amounts of phosphorylated H5 accumulate before chromatin condensation occurs. The accumulation of H5 and the timing of dephosphorylation appear to correlate well with genomic inactivation and chromatin condensation (Affolter et al., 1987; Mura et al., 1982).

There is not however a simple correlation between the amount of H5 expressed and the inactivation of the genome, we therefore sought to develop a functional assay which simulates the native physiological conditions so as to investigate further the biological role of H5. We have used a retroviral vector to introduce the H5 coding sequence into avian cells in which the endogenous gene is not expressed. This gene transfer method limits both the number of integrated copies and rearrangements of the integrated DNA (Hwang and Gilboa, 1984).

Retroviruses are now widely used for gene transfer because of their high efficiency both in penetrating the cell membrane and integrating their genome into the host cell DNA (Varmus and Swanstrom, 1982). Retroviral vectors in which the foreign inserted sequences are transcribed directly under the control of internal promoters are particularly interesting: they allow efficient translation of the transcribed RNA in the form of a native protein. Because of functional interference between viral and internal promoters (Emerman and Temin, 1984) a new generation of retroviral vectors

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has recently been developed, the so-called self inactivating (SIN)[1] vectors. These vectors carry mutations within their long terminal repeats (LTR) which inactivate transcription of the integrated provirus without affecting the expression from internal promoters (Yu et al., 1986). We developed an avian SIN vector to transfer the H5 coding sequence, linked to an internal promoter, into normal or transformed quail or chicken fibroblasts. We showed that expression of H5 in these cells inhibited the proliferation of normal fibroblasts, but surprisingly did not significantly affect the growth properties of transformed cells. Study of the posttranslational modifications of H5 showed that phosphorylation may be involved in this differential effect.

Materials and Methods

Construction of the Vector Genomes

The pDA1 genome was derived from the Rous-associated virus (RAV) 1 and 2 genomes. The pDAHY and pDAH5 were obtained from pDA1. All constructions were made by standard techniques (Maniatis et al., 1983). The cassette containing the enhancer and early promoter of simian virus 40 (SV40) and the bacterial neomycin phosphotransferase gene coding sequence (neo) were derived from the pSV_2Neo plasmid (Southern and Berg, 1982). The pDAHY was derived from pDA1 by inserting a cassette containing the bacterial hygromycin B phosphotransferase (hph) coding sequence (Gritz and Davies, 1983). This gene was put under the control of the enhancer and early promoter of SV40. The thymidine kinase (Tk) gene polyadenylation signal of Herpes simplex virus (HSV) was added at the 3' end. The pDAH5 genome was constructed from pDA1 by inserting a cassette containing the H5 coding sequence with its own polyadenylation signal, linked to the human metallothionein $MT\text{-}II_A$ promoter (Karin and Richards, 1982). The pMTH5 plasmid containing this cassette was kindly provided by Dr. A. Robins (University of Adelaïde, Adelaïde, South Australia). Construction of the pTXN3' vector was previously published (Benchaibi et al., 1989).

Cells

Chicken embryo fibroblasts (CEF) were prepared and grown as described previously (Gandrillon et al., 1987). Quail embryo fibroblasts (QEF) were prepared and grown in the same conditions. AEV-ES4 chicken cells, QT6 quail fibroblasts (Moscovici et al., 1977), and the G quail helper cells (Savatier et al., 1989) were grown in the CEF growth medium. AEV-ES4 cells were derived from CEF infected with the virus AEV-ES4 (Gandrillon et al., 1987); these cells were maintained in culture for several months before use. Selection for neomycin-resistant cell colonies was carried out in the presence of G418 (Gibco Laboratories, Grand Island, NY) at a concentration of 200 μg/ml.

Transfections and Infections

G helper cells were transfected by the polybrene-Dimethyl sulfoxide method (Kawai and Nishizawa, 1984). Virus suspensions were harvested over 12-h periods from subconfluent producer cells (3 ml per 100-mm dish), centrifuged for 15 min at 2,500 g, and then filtered through 0.22 μm low protein-binding filters (Millipore Continental Water Systems, Bedford, MA). Cells, seeded at 5.10^5 cells per 60-mm dish, were infected with 1 ml of virus solution for 12 h and then selected in G418. Resistant colonies were scored 10 to 15 d after infection. For infection of CEF by coculture, producer G cells were treated with mitomycin C (Sigma Chemical Co., St. Louis, MO) at 2 μg/ml for 2 h, washed three times, and then seeded at 2.10^6 cells per 100-mm dish over a layer of 2.10^6 CEF. Infected CEF were analyzed after 11 d in culture in the presence of G418. At this time, the mitomycin-treated producer cells showed no further protein production.

Growth Kinetics of Infected Cells

Cells were seeded at 5.10^5 cells per 60-mm dish and the medium was then changed every day. For each time point, one or three dishes per cell type were trypsinized and the cells counted.

DNA and RNA Analysis

High molecular weight DNA was extracted as previously described (Garcia et al., 1986). After digestion of 15 μg with appropriate restriction enzymes, the fragments were separated by electrophoresis in 0.8% agarose gels. DNA was then transferred onto nitrocellulose filters and hybridized with a random primed labeled probe. Filters were finally washed under high stringency conditions and exposed to Kodak-X-Omat film (Kodak-X-Omat; Eastman Kodak Co., Rochester, NY). The probe used was an EcoRI-XmnI fragment released from pMTH5, containing the H5 sequence.

Total cellular RNA was extracted after lysis of the cells in buffer containing 1-mM Dithiothreitol and 0.2% Nonidet P40 essentially as previously described (Garcia et al., 1986). S1-mapping experiments were carried out according to Favaloro et al. (1980). A 1,026 bp BamHI-PstI restriction fragment containing the H5 coding sequence was isolated from pMTH5 and labeled at the 5' end with (γ-^{32}P)ATP and polynucleotide kinase. The probe was coprecipitated with 10 μg of total RNA and resuspended in S1 hybridization buffer containing 40 mM Pipes (piperazine N-N' bis (2 ethanesulfonic acid, pH 6.4), 400 mM NaCl, 1-mM EDTA). Hybridization was performed for 6–9 h at 56°C and then hybrids were digested with 800 U of S1 nuclease per ml of digestion buffer (30 mM sodium acetate, pH 4.5, 250 mM NaCl, 2 mM $ZnSO_4$) for 30 min at 31°C. S1 nuclease-resistant DNA was finally run in a 6% polyacrylamide gel and exposed to Kodak-X-Omat film (Eastman Kodak Co.). The identity of the protected fragments was determined by comigration with appropriate molecular weight markers.

Electrophoretic Transfer of Proteins to Nitrocellulose Paper

Total histones were extracted with 0.4 M H_2SO_4 from purified nuclei. Pooled extracts were dialyzed and lyophilized. Histones were analyzed by SDS-PAGE (Laemmli, 1970). The electrotransfer from the polyacrylamide gel to nitrocellulose paper was done for 1 h at 24 V and the filters were finally incubated with anti-H5 antibodies and $1\text{-}10.10^5$ cpm ^{125}I-labeled protein A (Amersham Corp., Arlington Heights, IL). The production of antibodies in rabbits, immunized with H5 was previously reported (Mura and Stollar, 1981*a*). Anti-H5 antibodies were immunospecifically purified by affinity chromatography with H5 conjugated to Sepharose 4B. Their immunoreactivity was monitored by solid-phase radioimmunoassay (Mura and Stollar, 1981*b*).

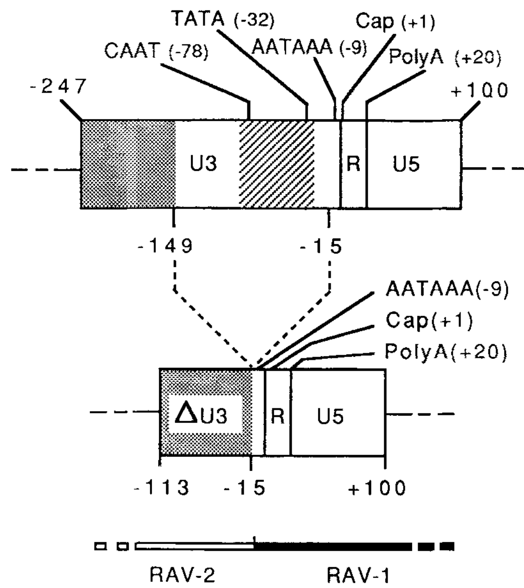
Immunofluorescence

The cells were grown in Lab-Tek Chamber slides (Miles Laboratories Inc., Elkhart, IN) and then fixed in absolute ethanol followed by acetone at room temperature. After washing with PBS-PMSF (10 mM sodium phosphate buffer, pH 7.4, 0.15 M NaCl, 0.1 mM PMSF), the cells were incubated for 30 min at 4°C with affinity purified anti-H5 antibodies. Fluorescein-labeled anti-rabbit antibodies were then added and stained cells were examined with a microscope (Olympus Corporation of America, New Hyde Park, NY) equipped with epi-illumination. Photomicrographs were taken with a camera (Olympus Corporation of America) and color film (Eastman Kodak Co.). Constant exposure and developing times were used in printing.

Phosphorylation Analysis of H5 Protein

Cells were incubated for 4 h in a phosphate-free minimal essential medium and then labeled for 4 h in the same medium with 2 mCi of ^{32}P-labeled H_3PO_4 (Amersham Corp.). Lysine-rich histones were extracted with 5% perchloric acid and an amount of labeled protein corresponding to 20,000 cpm was electrophoresed as described above. The phosphorylation of H5 was detected after transfer of the gel to nitrocellulose paper and autoradiography of the blot. The same blot was used for the detection of H5 protein with an anti-H5 antibody and protein A–horseradish peroxidase conjugate (Amersham Corp.). H5 was finally visualized by incubation in 10 mM Tris, pH 7.4, 0.025% H_2O_2, 0.04% 4-aminoantipyrin (Sigma Chemical Co.), and 0.2% phenol.

1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblasts; hph, hygromycin B phosphotransferase; HSV, Herpes simplex virus; LTR, long terminal repeats; Neo, neomycin; QEF, quail embryo fibroblasts; RAV, Rous-associated virus; SIN, self inactivating; SV40, simian virus 40; Tk, thymidine kinase.



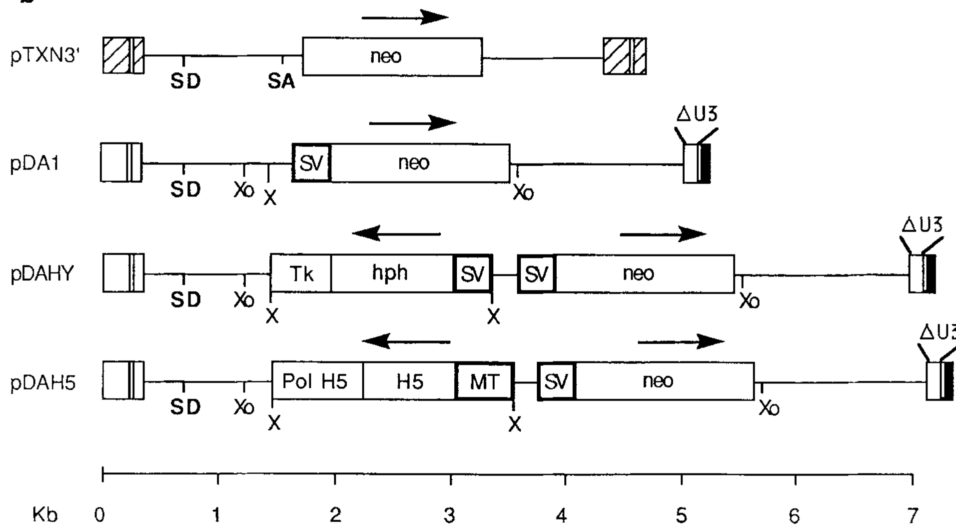


Figure 1. (*a*) Structure of normal (*top*) and deleted (*bottom*) LTRs. The localization of transcriptional regulation signals is indicated. The stippled and hatched areas in the U3 region represent, respectively, the enhancer and the promoter regions. The deleted U3 region (originated from RAV-2 proviral DNA) was linked, to the R and U5 regions of the RAV-1 LTR. The stippled lines indicate extent of the deletion. (*b*) Structure of retroviral vectors. In pTXN3', derived from AEV, the neomycin resistance gene (*neo*) is transcribed from the viral LTR, whereas in pDA1, it is transcribed from the internal SV40 promoter (*SV*). pDA1, originated essentially from RAV-2, harbors a deleted U3 region in the 3'LTR. pDAHY and pDAH5 were derived from pDA1 by inserting the hph (hygromycin resistance) and H5 genes, respectively. Arrows indicate the transcriptional orientation of the different genes. In pDAHY, the hph-coding region, fused to the polyadenylation site of the TK gene, was placed under the control of the SV40 promoter. In pDAH5, the promoter of the human metallothionein MT-II$_A$ (*MT*) was used to transcribe the H5 gene containing its own polyadenylation signal (*Pol H5*). AEV, RAV-2, and RAV-1 LTRs (▨, □, ■), respectively; splice donor and acceptor, (*SD* and *SA*), respectively; deleted U3 region, (*ΔU3*); XhoI, (*Xo*); XbaI, (*X*). The scale (in kilobase pairs) is given at the bottom.

Results

Construction of a Retrovirus Vector to Transfer and Express the H5 Coding Sequence

We constructed a mutated LTR by removing the U3 region isolated from the avian leukosis virus RAV-2 LTR between nucleotides −149 and −15 (relative to the transcriptional cap site at +1) (Fig. 1 *a*). This removed the CAAT and TATA boxes (Cullen et al., 1985; Laimins et al., 1984; Norton and Coffin, 1987) which were then ligated to the R and U5 regions isolated from RAV-1 LTR. The deleted LTR thus reconstituted was used to replace the normal 3'LTR in the vector pDA1. The pDA1 was constructed from RAV-2 and carries a transcriptional cassette containing the neo gene linked to the SV40 early promoter, inserted in the same transcription orientation as the viral transcription (Fig. 1 *b*). The pDAH5 vector genome was constructed from pDA1 by inserting a transcription cassette containing the H5 coding sequence with its own polyadenylation signal and fused to the promoter of the human metallothionein MT-II$_A$ gene. This promoter is regulated by heavy metals in human fibroblasts (Richards et al., 1984), but it was found to be constitutively activated without stimulation when inserted in the pDA1 vector and transduced in avian cells (data not shown). The H5 transcriptional cassette was inserted in the opposite orientation with respect to retroviral transcription. As a control virus, we constructed pDAHY, derived from pDA1 by inserting a cassette containing the hygromycin B phosphotransferase (hph) coding sequence between the SV40 early promoter

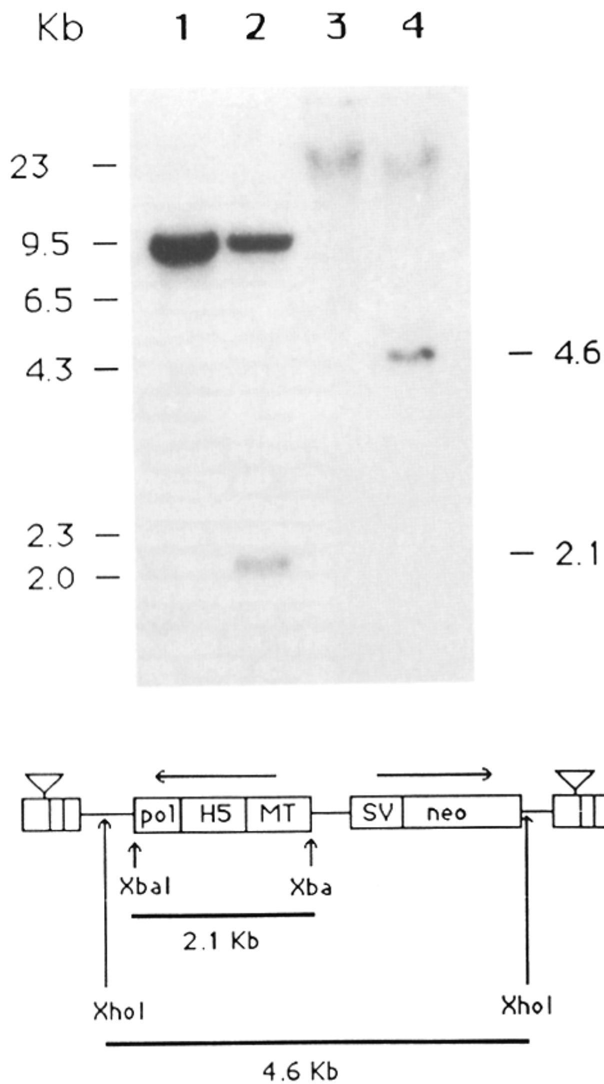


Figure 2. Southern blot analysis of QT6 cells infected with DAH5. 15 μg of DNA, isolated from infected (lanes *2* and *4*) and uninfected cells (lanes *1* and *3*), were digested with XbaI (lanes *1* and *2*) or XhoI (lanes *3* and *4*). After electrophoresis and transfer to nitrocellulose, the blot was hybridized with a H5 specific probe. Lambda DNA digested with EcoRI and HindIII was used as size marker. A schematic representation of the restriction map of DAH5 is drawn at the bottom.

and HSV Tk-polyadenylation signal. This transcription cassette was inserted in the opposite orientation relative to the neo gene. As an additional control, the virus pTXN3', a vector which expresses the neo gene from the viral LTR (Benchaibi et al., 1989) was used in parallel. All these retroviral genomes were transfected into the G helper cell line, which released only retroviral vectors free of helper virus (Savatier et al., 1989). The DAH5 helper-free virus was rescued with a titer of 10^2 rfu/ml (resistant forming U/ml) as determined by its ability to induce G418 resistance to recipient QT6 quail cells.

Analysis of Viral DNA and RNA in QT6 Cells Infected with DAH5

QT6 cells, a line of chemically transformed quail fibroblasts (Moscovici et al., 1977), were infected with a stock of helper-free DAH5 virus, then selected with G418 and amplified in polyclonal cultures. These cells did not release virus and the supernatants of the cultures, superinfected with a normal helper virus, were unable to transmit G418 resistance. Since no virus could be rescued, we conclude that in cells infected with DAH5, the integrated provirus is transcriptionally inactive. To assess the presence, organization and copy number of the introduced DNA vector, high molecular weight DNA from either infected or uninfected QT6 cells was prepared. 15 μg of DNA were digested with restriction enzymes and examined by Southern blot analysis with a probe containing the H5 coding region (Fig. 2). The restriction enzyme XhoI cuts twice within the vector and the presence of a 4.6-kb fragment is therefore characteristic of intact H5 and neo genes. The expected fragment of 4.6 kb was detected in infected cells (lane *4*) and was not present

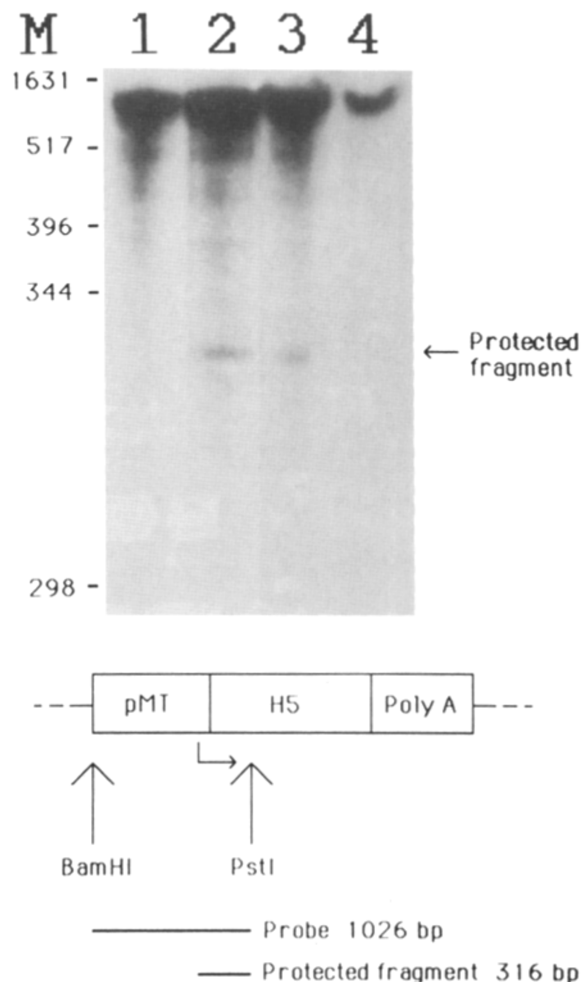


Figure 3. S1 nuclease protection assay of cytoplasmic RNA transcribed in QT6 cells. 20 μg of cytoplasmic RNA, isolated from uninfected cells (lane *1*) and from cells infected with DAH5 (lanes *2* and *3*), were hybridized to the H5 probe. In lane *3*, infected cells were maintained for 50 generations without G418 selection before extraction. A 1,026-bp fragment isolated from pMTH5 and representing the H5 probe (lane *4*) protected 316 nucleotides after S1 digestion. The numbers in the left margin represent the sizes, in nucleotides, of fragments used as molecular weight markers.

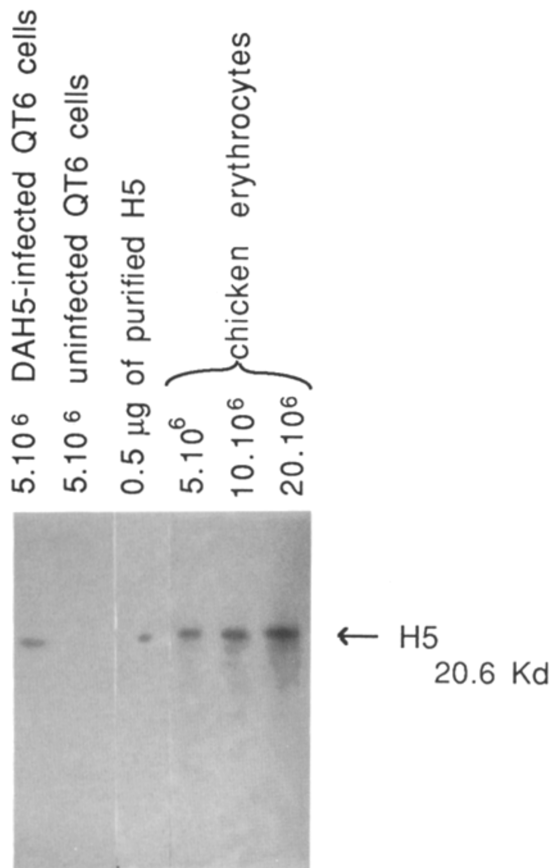


Figure 4. Western blot detection of H5 in QT6-infected cells. The nuclear proteins, isolated from 5.10^6 DAH5-infected QT6 cells or -uninfected QT6 cells, were electrophoresed in a SDS-polyacrylamide gel and transferred to nitrocellulose. H5 was detected after incubation with H5 antibodies and ^{125}I-protein A. 0.5 μg of purified H5 and total histones extracted from 5.10^6, 10.10^6, and 20.10^6 chicken erythrocytes were electrophoresed on the same gel.

in DNA from uninfected cells (lane *3*). To test more precisely for the presence of an intact MTII-H5 fragment, the DNA of infected and uninfected QT6 cells was cut with XbaI. As expected, a 2.1-kb fragment was detected in infected cells (lane 2) and not detected in uninfected cells (lane *1*). These data show that the inserted proviruses did not undergo major rearrangements and exhibited the expected structure. Fragments of higher molecular weight seen in infected and uninfected cells correspond to the endogenous H5 gene. From the relative intensities of bands at 2.1 and 9.5 kb in infected cells we estimated that one or a limited number of exogenous H5 gene had integrated per cell genome. This estimation was confirmed by comparing the intensity of the 2.1-kb band with that given by known amounts of H5 coding sequences (data not shown).

To determine the levels of specific H5 gene transcripts in DAH5 infected and uninfected QT6 cells, a quantitative S1 nuclease analysis was used (Fig. 3). The DNA probe was a 1,026 bp BamHI-PstI fragment isolated from the pMTH5 plasmid. This fragment contains the MT-II$_A$ promoter and 216 nucleotides of the H5 coding sequence. Transcripts initiated at the MT-II$_A$ promoter should be protected over a length of 316 bp (Karin and Richards, 1982). As expected, a protected fragment of 316 nucleotides was detected with RNA from DAH5 infected QT6 cells (lane *2*) and not with RNA from uninfected QT6 (lane *1*). A protected fragment of the same size was detected in DAH5 infected QT6 cells maintained in culture for 50 generations without G418 selection (lane *3*). From this we can conclude that the transcription of the H5 coding sequence was efficiently and correctly initiated in QT6 cells and that the H5 coding sequence once transferred into the QT6 cells was expressed stably for at least 50 generations in cultures released from G418 selection.

Expression of H5 Protein in QT6 Fibroblasts Infected with DAH5 Virus

The synthesis of histone H5 was measured in DAH5 infected and uninfected QT6 cells. Total histones were extracted from purified nuclei isolated from the respective cells. Proteins were analyzed by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and the presence of H5 was detected after incubation of the blot successively with an anti-H5 antibody and ^{125}I-Protein A (Fig. 4). This antibody did not give any signal when assayed against total histones from uninfected QT6 cells. A clear visible signal with the same mobility of purified H5 or H5 from chicken erythrocyte total histones, was detected with total histones from 5.10^6 DAH5-infected QT6 cells. In an attempt to quantify the production of H5 in infected QT6 cells, total histones extracted from 5.10^6, 10.10^6, or 20.10^6 adult chicken erythrocytes were electrophoresed on the same gel. From these results we can conclude that the amount of H5 present in 5.10^6 DAH5-infected QT6 cells is equivalent to the amount of H5 produced by 5.10^6 adult chicken erythrocytes. The nuclear localization

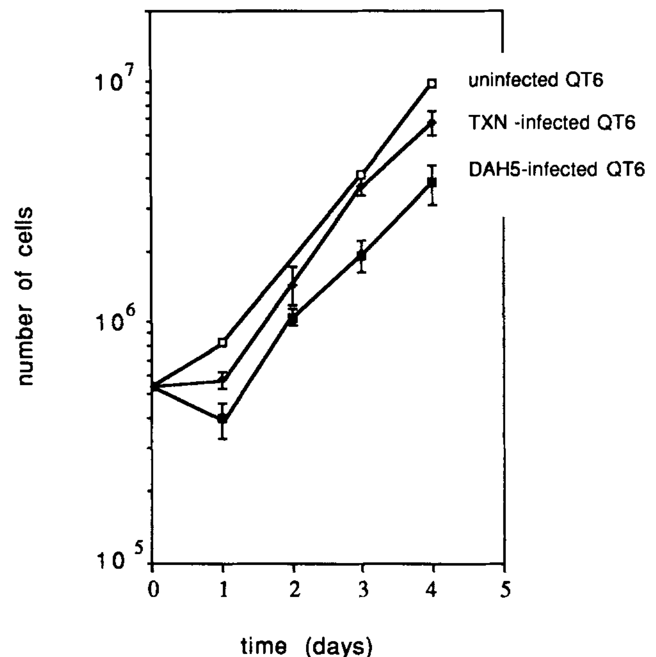


Figure 5. Growth kinetics of QT6 cells. Cells infected with DAH5 were compared to cells infected with TXN3′ and to uninfected cells. For infected cells, each point represents an average value ± SD from three different dishes.

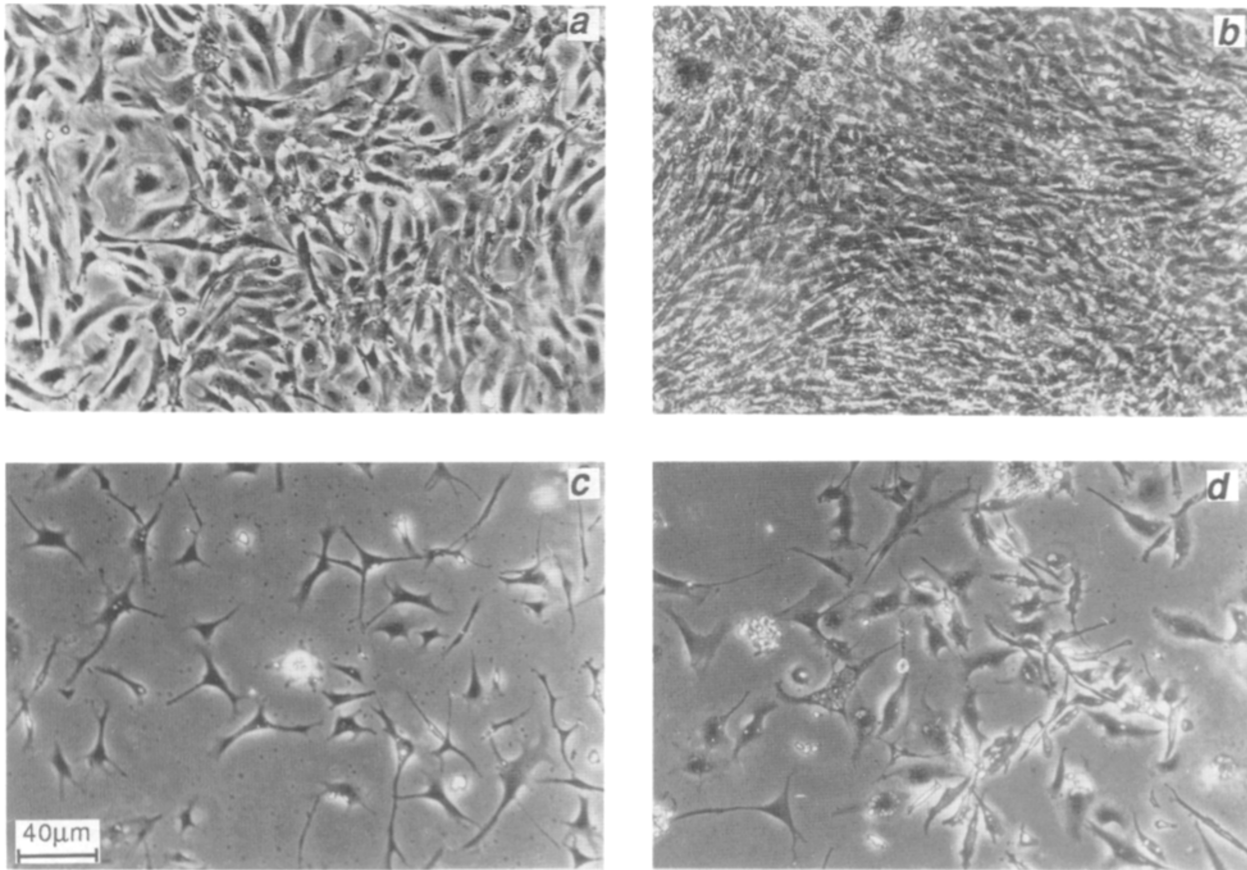


Figure 6. Comparison of the phenotype of uninfected and DAH5-infected fibroblasts. Uninfected cells are shown in *a* and *b*; infected cells in *c* and *d*. *a* and *c* show quail fibroblasts whereas *b* and *d* show chicken fibroblasts.

of H5 in the DAH5-infected QT6 cells was confirmed by immunofluorescence (data not shown).

Properties of QT6 and AEV-ES4 Transformed Fibroblasts Expressing H5 Protein

QT6 cultures infected with DAH5 developed large dense foci containing cells phenotypically not obviously different from uninfected QT6 cells or QT6 cells infected with TXN3′ control virus. Similarly, chicken fibroblasts transformed by the avian retrovirus AEV-ES4, were infected with DAH5. These cells developed foci indistinguishable from those infected with TXN3′ and could be further expanded. To further investigate whether the H5 protein could have some effect on the growth of transformed cells, we compared the growth kinetics of uninfected QT6 cells with those of QT6 infected with either DAH5 or TXN3′. The three cell types were individually seeded at the same density in 60-mm dishes and cultured under the same conditions. At different times thereafter the cells were trypsinized and counted. The growth curves are presented in Fig. 5. It can be seen that the QT6 cells infected with DAH5 exhibited a longer latency period and a slower growth than uninfected or TXN3′-infected QT6 cells. The generation times could be estimated as 20 h for control QT6 cells and 25 h for QT6 cells producing H5 protein. We can conclude then, that the expression of H5 protein seems to have only a small effect on the growth of QT6 cells and fibroblasts transformed by AEV-ES4.

Expression and Effects of H5 Protein in Normal Fibroblasts

CEF and QEF were infected with either DAH5 virus or the control viruses, TXN3′, or DAHY. 10 d after selection in medium containing G418, large dense clones of fibroblasts were observed in the quail and chicken cultures infected with each control virus (Fig. 6, *a* and *b*). In all cultures infected with DAH5 only few small clusters of sparse cells were observed (Fig. 6, *c* and *d*). These cells grew very slowly and could not be efficiently replated and therefore could not be amplified. In addition, most of these cells looked ailing and displayed a starlike flat morphology identical to quiescent fibroblasts. The same observations were made in six independent experiments using either chicken or quail secondary fibroblasts. Since cells infected with DAHY control virus looked as healthy as normal fibroblasts, the effects observed with DAH5 virus are most likely because of the expression of the H5 coding sequence. The expression of the H5 protein in these cells was analyzed by immunofluorescence (Fig. 7, *b* and *d*), using Giemsa-Wright staining of the same field to distinguish nuclei and cytoplasm (Fig. 7, *a* and *c*). Normal chicken fibroblasts infected with DAH5 showed heterogeneous fluorescence when treated with anti-H5 (Fig. 7 *d*). All the cells displayed fluorescent nuclei and the intensity of the fluorescence correlates well with the structure of the chromatin (Fig. 7 *c*). Highly condensed nuclei showed the brightest fluorescence, while the nuclei with a less-condensed

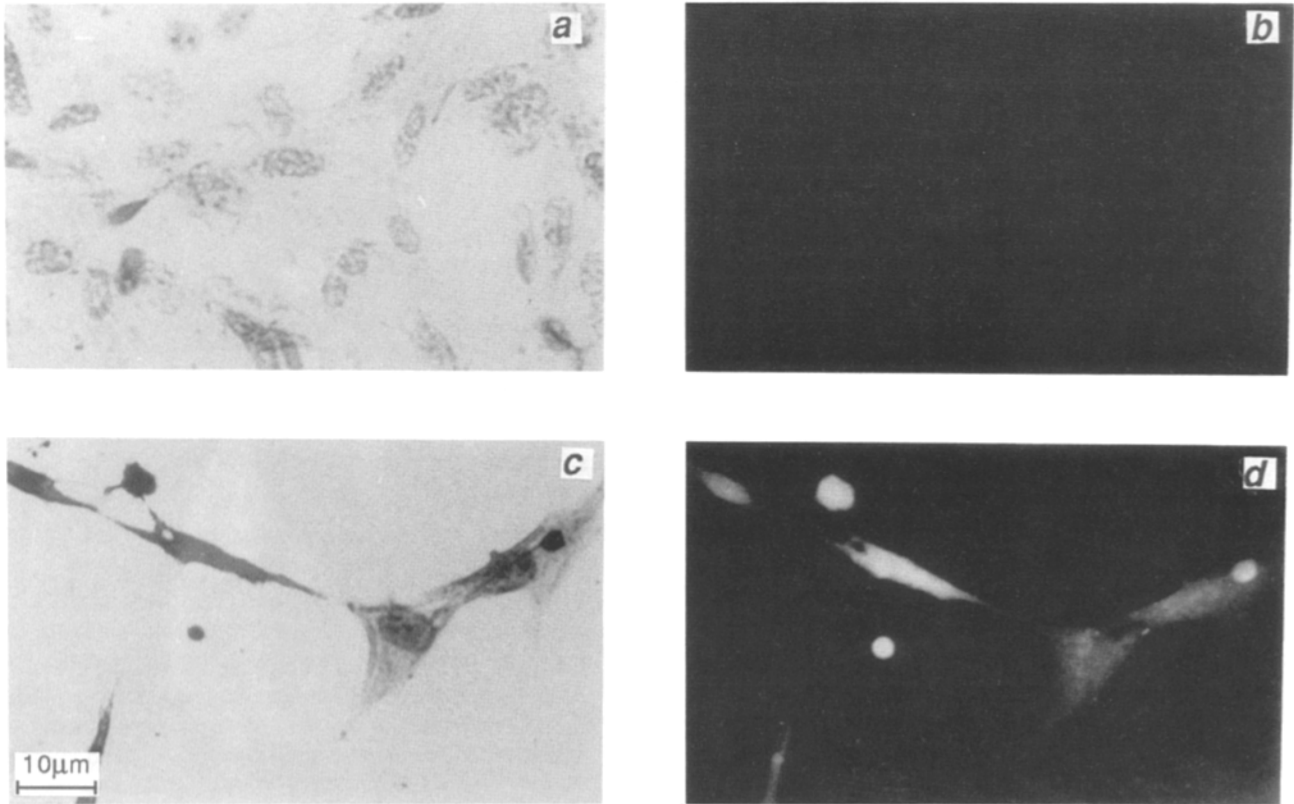


Figure 7. Localization of H5 in DAH5-infected CEFs by indirect immunofluorescence. After the IF reaction (*b* and *d*), the cells were stained with Wright-Giemsa (*a* and *c*), and the same microscopic fields were photographed. *a* and *b* represent uninfected fibroblasts and *c* and *d* show DAH5-infected primary fibroblasts.

chromatin showed dim fluorescence. As shown in Fig. 7 *d*, some highly condensed nuclei seemed to be extruded from the cells. Conversely, uninfected normal fibroblasts did not exhibit significant fluorescence (Fig. 7 *b*) and contained nuclei with typical dispersed chromatin (Fig. 7 *a*).

H5 Is Phosphorylated in Transformed QT6 Cells and not in Normal Fibroblasts

It appears from our results that expression of the H5 protein has different effects on the growth of transformed and normal cells. We therefore looked for possible posttranslational modifications of H5 that might lead to this difference. Since phosphorylation may be involved in the inactivation of H5 in early stages of normal erythropoiesis (Sung et al., 1977), we compared the extent of phosphorylation of H5 in CEF and QT6 cells. Since we had many difficulties amplifying the DAH5-infected CEF, we increased the number of infected cells per dish by co-cultivating CEF with the DAH5-producer G cells previously treated with mitomycin C. This treatment inhibits replication without interfering with viral production. DAH5- and TXN3′-infected CEF and QT6 cells were incubated in the presence of ^{32}P-labeled H_3PO_4. The lysine-rich histones were then extracted, and an amount of labeled protein corresponding to 20,000 cpm was electrophoresed in an SDS–polyacrylamide gel, transferred to nitrocellulose and the extent of phosphorylation was directly analyzed by autoradiography of the blot (Fig. 8, *AUTORADIO*). The pattern of phosphorylation shown by DAH5-infected CEF was identical to that of control TXN3′-infected CEF. For QT6 cells however, a faster migrating phosphorylated protein absent from the control cells (as well as from infected CEF), was detected in DAH5 infected QT6 cells. To identify this protein, the same blot was treated with an anti-H5 antibody, protein A–peroxidase conjugate and 4-aminoantipyrin as substrate (Fig. 8, *ANTIBODY DETECTION*). H5 protein was detected in DAH5-infected CEF, in DAH5-infected QT6 cells and in lysine-rich histones extracted from chicken erythrocytes, no band was observed in control CEF and QT6 cells. To demonstrate protein mobilities, the chicken erythrocyte lysine-rich histones blot was stained with ponceau red (Fig. 8, *STAIN*). The phosphorylated protein detected only in DAH5-infected QT6 cells has the same mobility as the H5 protein, whilst the phosphorylated proteins present both in transformed (one band) and in normal fibroblasts (two bands) correspond to H1 histones. Thus, we can conclude that the H5 protein is phosphorylated in DAH5-infected QT6 cells but is not phosphorylated in DAH5-infected CEF. This is in contrast to H1 which is phosphorylated in both normal and transformed fibroblasts.

Discussion

To study the effect of the expression of H5 on the phenotype of cells which do not express this protein, we sought to obtain long-term and stable expression of the protein. The present work demonstrates the efficient transfer and expression of the histone H5 gene into normal and transformed fibroblasts, using a retroviral SIN vector, which was con-

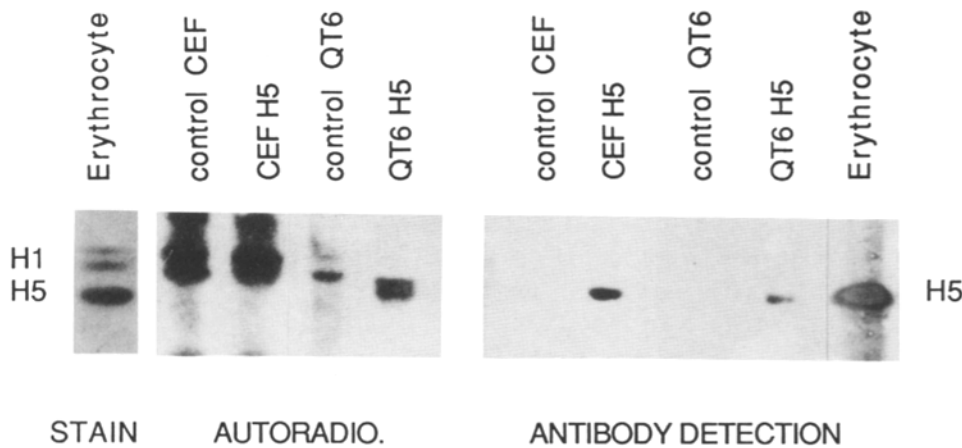


Figure 8. Phosphorylation of H5 in DAH5-infected CEF and QT6 cells. Lysine-rich histones were extracted from TXN3′ (control CEF) and DAH5 infected CEF (CEF H5), and from DAHY (*control QT6*) and DAH5-infected QT6 cells (*QT6 H5*) after incubation with [^{32}P]phosphate. Histones were electrophoresed on SDS-polyacrylamide gel, together with lysine-rich histones extracted from chicken erythrocytes. [^{32}P]-labeled proteins were analyzed directly after transfer and autoradiography of the blot (*AUTORADIO*). The identification of the H5 protein in this blot was done with an anti-H5 antibody, protein A-peroxidase conjugate and 4-amino antipyrin as substrate (*ANTIBODY DETECTION*). In addition, lysine-rich histones of chicken erythrocytes were visualized by staining of the blot with red ponceau (*STAIN*).

structed from the avian leukosis virus RAV-2, by deletion of the promoter sequences in the 3′LTR. This mutation leads to integration of an inactive "provirus" in the infected cells. In our conditions of infection, the vector transferred a very low number of copies of the foreign H5 sequence into the genome of each infected cell. These transformants were found to be highly stable since the inserted H5 sequence was still present in cells grown for 50 generations without selective pressure. As expected, in cells infected with the DAH5 SIN vector, the H5 sequences were transcribed from the internal MT-II$_A$ promoter. Since no vector virus could be rescued from the infected cells after superinfection with a helper virus, it is likely that no transcription was initiated from the 5′LTR. The SIN vector described here is thus quite reliable and efficient.

The amount of H5 protein produced per QT6 cell infected with DAH5 is similar to that found in mature avian erythrocytes and showed exclusively nuclear localization. The effects of H5 in the infected cells can therefore be compared with those under native physiological conditions.

Expression of H5 in transformed cells, i.e., QT6 quail fibroblasts or chicken embryo fibroblasts transformed by AEV-ES4, did not greatly inhibit their growth in vitro. QT6 infected with DAH5 exhibit only a longer latency period and a slight increase of their generation time, from 20 to 25 h, but their ability to grow as an established line was not impaired. This result demonstrates explicitly that the constant production of H5 has little effect on the phenotype of transformed avian fibroblasts.

In contrast, normal quail or chicken fibroblasts expressing the exogenous H5 protein displayed a very limited growth potential and could not be replated and expanded. Moreover, these cells look similar to quiescent cells with flat morphology, vacuolated cytoplasm, and condensed nuclei. The condensation of the chromatin in these cells was directly correlated with the expression of H5 since the more condensed nuclei showed the brightest fluorescence when treated with the anti-H5 antibody. In addition, some nuclei with highly condensed chromatin were extruded from the cells.

Comparison of ^{32}P-labeled DAH5-infected CEF with DAH5-infected QT6 shows that H5 is highly phosphorylated in infected QT6 cells as well as in erythroid cell lines such as 6C2 (this work, and data not shown) and is not phosphorylated in infected normal fibroblasts. This mimics the process of normal erythropoiesis in which the presence of H5 has been detected at reduced level in the early dividing erythroblasts. In these cells, the newly synthesized protein becomes modified through a process during which nine phosphoryl groups may be introduced. At the terminal stages of red cell maturation, the phosphorylated protein becomes dephosphorylated and this last event correlates well with the cessation of DNA and RNA synthesis and chromatin condensation (Briand et al., 1980; Sung and Freelender, 1978).

The biological role of H5 has frequently been linked to the idea of being a general repressor. This view was supported by the findings that when chicken erythrocyte nuclei were reactivated by fusion with HeLa cells, early events include decondensation of chromatin, initiation of RNA synthesis, and disappearance of H5 (Appels et al., 1974). Two recent studies emphasizing the repressive role of H5 are particularly demonstrative. The first, microinjection of H5 into proliferating L6 rat myoblasts (a cell line that maintains the potential to differentiate into myotubes) showed that the migration and localization of H5 in nuclei was linked to a decrease in cell size, vacuolization of the cytoplasm, compaction of the nuclei, and inhibition of transcription and replication (Bergman et al., 1988). The second study reported the inhibition of replication and arrest of cells in G1 after transfection and transient expression of the H5 gene in rat sarcoma XC cells (Sun et al., 1989, 1990). These effects of genomic repression in L6 and XC cells are similar to those reported here for normal avian fibroblasts, although L6 and XC cells can be regarded as transformed cell lines. In those two reports only one cell system was studied and the protein was expressed transiently, i.e., in sarcoma XC cells, the amount of H5 begun to decrease after 48 h and mRNA was absent at 120 h after induction, in contrast with the long-term expression of H5 in QT6 cells. It is possible therefore that the inhibition of replication they observe may be consistent with the increase of latency period and generation time we have detected on the transformed cell line. In fact, QT6 cells grew slower than normal cells. What we have shown is that the effect of the H5 expression in both normal chicken and quail fibroblasts is much more dramatic than the effect

in the transformed cell lines derived from them, chicken fibroblasts transformed by AEV-ES4, and quail fibroblasts by chemical means (QT6). Most relevant is the observation that H5 expressed in transformed cells (QT6 and the erythroid cell line 6C2) is phosphorylated in contrast to normal cells. If one concludes that phosphorylation of H5 prevents the condensation of chromatin, one must also conclude that H5 expressed in L6 and XC cells is not phosphorylated. The magnitude of the effect of H5 in different cell lines however may depend not only on whether the protein is phosphorylated but also on the degree of phosphorylation. Up to nine sites of phosphorylation have been described in H5 and its degree of phosphorylation was found to be proportional to the number of immature cells during avian erythropoiesis (Sung, 1977).

The exact mechanism of interaction of H5 with DNA in chromatin is not known. However, we can visualize a strategy that allows the cell to handle high amounts of H5 without impairing the cell metabolism. The phosphorylation of H5 produces a protein that has less affinity for DNA, through neutralization of charges. Therefore, the modified amino acids should be located in domains which are crucial for this interaction, i.e., the COOH-terminal region (residues 100–182) which is the region that contributes most to chromatin condensation through partial neutralization of DNA phosphates. The unmodified H5 showing more affinity for higher order structures of chromatin and DNA, would bind tightly to DNA, perhaps to A-T rich regions, condensing and/or changing its conformation (Mura and Sollar, 1984), and in this manner, blocking the binding of transcription and replication machinery.

It will be relevant to investigate the metabolic pathways that are directly responsible for the phosphorylation of H5 in transformed cells and their relationship with oncogene expression. Indeed, the crucial features of the cell cycle are the two transition control points at G2M and Gl phase. M phase is characterized by the activation of an Hl kinase. In our normal fibroblasts the expressed H5 was observed not to be phosphorylated while Hl was phosphorylated (Fig. 8). This suggests that the cdc2 kinase is not responsible for the phosphorylation of H5.

The analysis of H5 modifications in various cells infected with DAH5 may be helpful in defining the role of this protein in cell replication and differentiation, and in understanding the role of phosphorylation in oncogenic transformation.

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