In vivo incorporation of *Drosophila* H2a histone into mammalian chromatin

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Received 20 January 1983; Revised and Accepted 6 April 1983

ABSTRACT

Hybrid prokaryotic/eukaryotic expression vectors have been used to introduce *Drosophila* histone genes into CV-1 African green monkey tissue culture cells. Transfection of CV-1 cells with *Drosophila* genes under the control of insect DNA promoter sequences results in low level expression of histone genes. On the other hand, when the *Drosophila* H2a gene is juxtaposed downstream from the long terminal repeat sequence of Rous sarcoma virus (RSV) expression of the insect gene is considerably more efficient; both 3' polyadenylated insect histone messenger RNA and putative *Drosophila* H2a histone protein can be readily detected in the transduced cells. Using this RSV/H2a vector, we have been able to demonstrate the presence of *Drosophila* H2a histone in monomer nucleosome preparations isolated from transfected CV-1 cells. These results suggest the feasibility of 'remodeling' cellular chromatin *in vivo* in precisely defined ways. The techniques described may be generally applicable to other genes coding for chromosomal proteins.

INTRODUCTION

Chimeric recombinant molecules of simian virus 40 (SV40) have proven to be efficient vehicles for the introduction of foreign DNA into mammalian cells (1). Many types of introduced genes (whether of eukaryotic (2-5), prokaryotic (6-11), or viral (12) origin) carried on such hybrid vectors have been demonstrated to function in recipient transduced cells. Indeed, in certain cases the expression of the foreign genes has been made the basis for selection of 'dominant' genetic markers in cultures of mammalian cells (5-9). We wished to determine whether foreign histone genes would be functional when they were introduced into cultured mammalian cells as part of various hybrid SV40 expression vectors. Our eventual aim in such studies was to develop a system whereby mammalian cell chromatin could be 'remodeled' *in vivo*. Such an ability could potentially allow for a more detailed study of the relationship of chromatin structure and function than is currently possible (13,14).

The cloned H2a and H2b histone genes of *Drosophila melanogaster* (15,1

have been used as a model system for these studies. These insect genes have been ligated into derivatives of the SV40 expression vector pSV2 (1,7,11) and then introduced into CV-1 African green monkey kidney tissue culture cells by the calcium phosphate precipitation method (17). The recipient cells were monitored for the presence of insect histone messenger RNAs and authentic insect histone proteins. Our results demonstrate that the Drosophila genes introduced into CV-1 cells do function, although the efficiency is dependent on the type of vehicle constructions used in the cell transfections. Specifically, when the H2a histone gene was placed under the promoter control of the Rous sarcoma virus long terminal repeat (LTR) sequence (11,18) the gene was efficiently expressed. Using this vector construction we have been able to detect putative Drosophila H2a histone in isolated monomer nucleosomes obtained from transfected CV-1 cells. These results demonstrate the feasibility of using existing techniques to introduce foreign proteins into mammalian chromosomes in vivo.

MATERIALS AND METHODS

Cell Cultures and DNA Transfections:

Methods used for the routine maintenance and DNA transfections of both CV1 monkey kidney tissue culture cells and bacterial cells have already been described (7,10). For isotopic labeling, CV1 cells were grown in normal medium (90% Dulbecco's Modified Eagle's Medium plus 10% fetal calf serum) lacking the non-radioactive compound used for radioisotopic labeling. Radioisotopes were obtained from New England Nuclear.

Plasmid DNAs used for the calcium phosphate precipitation method of mammalian cell transformation (17) were isolated and purified by two consecutive cesium chloride-ethidium bromide equilibrium centrifugation steps (10). The procedures used in both the transfection of plasmids into CV1 cells and the assay of recipient cells for the presence of chloramphenicol acetyltransferase enzyme activity (CAT assays) have been described (10).

Enzymes and Recombinant DNA Methods:

Restriction endonucleases were obtained from New England Biolabs or Bethesda Research Laboratories and were used according to the supplier's specifications. DNA polymerase I was obtained from Boehringer-Mannheim, T4 polynucleotide kinase from P-L Biochemicals, bacterial alkaline phosphatase from Worthington Diagnostics or New England Biolabs and T4 DNA ligase from New England Biolabs. Details of the recombinant DNA procedures used for the construction of various plasmids have been described (2,10,11).

Isolation and Electrophoretic Separations of Histones:

Total nuclear histones from either mammalian (CV1) or *Drosophila* embryo (Schneider) tissue culture cells were isolated by previously described methods (46). The intermediate lysine-rich histones (principally the H2a and H2b groups) were selectively extracted from CV1 nuclei using the method of Ohlenbush et al. (27). Briefly, isolated nuclear chromatin was first extracted with a buffer solution containing 0.8 M NaCl to remove the H1 histones and various non-histone proteins and then pelleted by centrifugation. The pellet was then extracted three times with a buffer solution containing 1.2 M NaCl, the extracts pooled and dialyzed against water, and finally lyophilized.

Histones were separated by the electrophoretic method of Zweidler (24) using 15% polyacrylamide gels containing 5% acetic acid, 7.5 M urea and the non-ionic detergent Triton X-100 at 6 mM (AUT gels). Identification of the various histone species on such AUT gels was based both on published reports (23-26) and on unpublished control experiments. Cyanogen bromide cleavage of electrophoretically purified *Drosophila* H2a histones was by published procedures (29) and the peptide fragments separated by electrophoresis on 25% polyacrylamide gels containing SDS (46). Fluorography was by the method of Laskey and Mills (28).

Analysis of RNAs Produced by Recombinant Genomes:

Total cellular RNA was extracted from CV1 cells by published procedures (38) and separated into poly (A⁺) and poly (A⁻) fractions by chromatography on oligo-(dT) cellulose as described by Aviv and Leder (39). For RNA 'Dot-Blot' hybridization assays, RNA was covalently bound to activated diazobenzyl-oxyethyl (DBM) paper by the methods described by Alwine (45) and hybridizations performed as described previously (38). Isolated DNA fragments used for hybridizations were labeled at their 5' termini using γ -(³²P)-ATP and T4 polynucleotide kinase (31).

Two different methods were used to determine the 5' terminal start site for H2a gene transcription in plasmids: S1 nuclease mapping (30,31) and in vitro primer extension (32). Radioactive DNA restriction fragments were isolated from agarose gels by the method of Chen and Thomas (20). The method of Southern (33) was used to transfer electrophoretically separated DNA fragments from agarose gels to nitrocellulose filter for hybridization studies.

Nucleosome Isolation and Characterization:

Chromatin fragments were released from isolated CV1 nuclei by digestion with micrococcal nuclease (Sigma) followed by separation of the monomer nucleosome species by centrifugation on a 10-30% sucrose density gradient by

Figure 1: Structure of *Drosophila* Histone Gene Transducing Vectors. The vectors are derivatives of pSV2cat (10) and pRSVcat (11). pBR322 DNA is represented by the solid black segments and contains the pBR322 origin of DNA replication and the B-lactamase gene (Amp^R). SV40 viral DNA segments are clear with the origin of SV40 replication (ori) indicated. The bacterial gene coding for chloramphenicol acetyltransferase (CAT) is cross-hatched and those DNA sequences coding for the *Drosophila* histone proteins are hatched. The stippled areas represent non-coding insect DNA sequences between adjacent *Drosophila* histone genes. The area with wavy lines is a 524 bp fragment of the Rous sarcoma virus long terminal repeat (LTR) which contains a start site ($\blacktriangle$) for promotion of RNA transcription (11,18). Some important restriction enzyme cut sites are indicated and those enclosed in parentheses represent a previous cut site for that enzyme which was lost during vector construction. The region of SV40 DNA between the Sau 3A (Bgl II) and Bam HI restriction sites contains the small tumor antigen intervening sequence and splice site and the 3' polyadenylation site for the early SV40 transcripts (1). The open arch represents the 1.85 kb Hinf I restriction fragment isolated from plasmid cDM500 which contains the *Drosophila* H2a and H2b genes and a small fragment of the 3' end of the H1 histone gene (16). After blunting of the fragment ends and the addition of synthetic Bam HI oligonucleotide linkers, the DNA fragment was ligated into the unique Bam HI site of the vector pSV2cat (10). Both orientations of the *Drosophila* H2a and H2b genes were recovered in recombinant constructs and were, individually, used in the mammalian cell transfection experiments described here.

described methods (38). Electrophoretic separations of chromatin fragments on 1.4% agarose gels and the visualization of the fragments by ultraviolet light after staining with ethidium bromide were by published procedures (46).

RESULTS

Construction of Histone Gene-Containing pSV2 Vectors:

Two different recombinant derivatives of pSV2 (1,6,10) were used as vehicles for the introduction of *Drosophila* histone genes into CV-1 monkey cells (Fig. 1). The construction of one of these, pSV2cat, has been described in detail elsewhere (10). This vector contains the bacterial gene (obtained

from E. coli transposable element Tn9) coding for the enzyme chloramphenicol acetyltransferase (CAT) inserted between the unique Hind III and Bgl II restriction sites in the parent pSV2 plasmid (10). The CAT gene is controlled by the SV40 early promoter region and is expressed efficiently in various types of transfected mammalian tissue culture cells (10). The Drosophila H2a and H2b histone genes were obtained from the col E1 derivative plasmid cDm500 (15,16) carried on a 1.85 kb Hinf I restriction fragment to which synthetic Bam HI oligonucleotide linkers were added (2). After cleavage with Bam HI, the histone gene-containing fragment was ligated (2) into the unique Bam HI restriction site of the vector pSV2cat resulting in a recombinant molecule, pSV2cat-DmH2a/b, of 6.8 kb (Fig. 1). Both orientations of the H2a and H2b histone genes (relative to the SV40 origin region) were recovered in the recombinants. In these constructions the insect genes are under the control of their endogenous promoter and terminator sequences, although the level of their expression could be affected by the SV40 'enhancer' sequences present in these plasmids (19,47).

The second pSV2 derivative used, pRSV-DmH2a, contained only the Drosophila H2a histone gene sequence under the promoter control of the Rous sarcoma virus long terminal repeat (LTR) region (11,18) (Fig. 1). In the plasmid pRSV-DmH2a, the isolated H2a gene replaces the bacterial CAT gene sequence in the parent vector pRSVcat (11). In this plasmid, pRSV-DmH2a, the histone H2a gene is under promoter control of the RSV-LTR. The transcriptional 'start site' of the Rous LTR is positioned about 270 bp 5' upstream from the AUG initiation codon of the H2a gene (see the diagram in Fig. 5).

Transfection of CV-1 Cells with pSV2cat-DmH2a/b:

Early published reports suggested that the Drosophila histone genes contained on the col E1 plasmid cDm500 (15,16) might function in the synthesis of both messenger RNA (21) and a putative "H2a-like" histone protein (22) when microinjected into the nucleus of Xenopus oocytes. Although the level of expression was rather low in the oocytes compared to other genes, these results suggested that the Drosophila genes contained their own promoters for initiation of histone messenger RNA transcription (21). More extensive work on sea urchin histone genes microinjected into Xenopus oocytes, however, has shown that sea urchin histone genes contain their own gene-specific regulatory sequences and that the genes are efficiently transcribed in this system (34-37). Encouraged by these results, we ligated the Drosophila H2a and H2b genes into the vector pSV2cat in a position where the insect genes would be under their own endogenous transcription control sequences, albeit

Figure 2A: Assay of CAT (chloramphenicol acetyltransferase) Enzyme Activity in Transfected CV-1 Monkey Cells. Autoradiograph of a thin layer chromatograph on which the various forms of (^{14}C)-chloramphenicol found in different CV-1 cell extracts have been separated (10). Lane (1): The reference standard (^{14}C)-chloramphenicol (unacetylated); The remaining lanes show the products of chloramphenicol found in extracts of cells forty-eight hours after the cells have been transfected with the following types of DNAs: Lane (2): pSV2cat; Lane (3): pSV2cat-DmH2a/b; Lane (4): pSV0cat (a construct which lacks the SV40 ori region (17)); Lane (5): control CV-1 cells transfected with calf thymus DNA. **Figure 2B: Turnover of Histone Messenger RNA in CV-1 Cells.** Forty-eight hours after cell transfection with either control, pSVcat, plasmid DNA or with experimental, pSV2cat-DmH2a/b, plasmid DNA, cell cultures were labeled for 4 hr with (^{32}P)-phosphate at a concentration of 100 $\mu\text{Ci}/\text{ml}$ as a "pulse". The cells were washed three times in isotope-free medium containing "cold" phosphate (10 mM) and returned to normal growth medium and incubated at 37° for the times indicated during a "chase". At the times indicated, total cellular RNA was

isolated from cells and separated into poly(A⁺) and poly(A⁻) fractions by affinity chromatography. The various RNA fractions were hybridized to an excess concentration of isolated *Drosophila* H2a and H2b histone gene DNA (the Bam HI fragment of pSV2cat-DmH2a/b) and S1 nuclease-resistant hybrids (44) isolated and counted for radioactivity. LEGENDS: (●—●), hybridizable poly(A⁻) RNA from pSV2cat-DmH2a/b transfected cells; (○—○), hybridizable poly(A⁻) RNA from pSVcat transfected cells (controls); (■—■), hybridizable poly(A⁺) RNA from pSV2cat-DmH2a/b transfected cells; (□—□), hybridizable poly(A⁺) RNA from pSVcat transfected cells (controls).

possibly influenced by the SV40 enhancers (19,47). Additionally, the presence of the bacterial CAT on the same vector (under the control of the SV40 early promoter region (11)) allowed for an internal standard.

Figure 2 shows the results of transfecting CV-1 monkey kidney cells with purified pSV2cat-DmH2a/b plasmid DNA using a modification of the calcium phosphate precipitation method (10,17). Forty-eight hours after transfection, extracts of the cells were prepared and assayed for CAT enzyme activity (10). Panel A of Figure 2 demonstrates that the CAT gene is active in the transfected cells since assays show readily detectable amounts of monoacetylated forms of radioactive chloramphenicol in the experimental (channel 3), but not control (channel 5), cell extracts.

The transfected cells were next analyzed for the presence of insect histones. Forty-eight hours after transfection, cells were labeled for four hours with (¹⁴C)-methionine (2 uCi/ml). Nuclear histones were isolated and analyzed by electrophoresis on acid-urea-triton (AUT)-containing polyacrylamide gels (23,25). It is possible to distinguish the *Drosophila* H2a and H2b histones from the analogous CV-1 cell histones by their differing electrophoretic mobilities on AUT-gels (23), however, in these initial experiments we failed to detect bona fide insect histone proteins in the transfected cells even though the *Drosophila* histones contain methionine (16,23). If the transfected cells are labeled for longer periods of time (8-12 hours) with this same isotopic compound at much higher concentrations (20 uCi/ml or greater), low concentrations of *Drosophila* H2a histone protein could be detected in these cells (unpublished data). The above results demonstrate that at least one of the introduced insect histone genes is functional in the mammalian cells but the efficiency is low under these experimental conditions.

Comparison of the RNA species synthesized in CV-1 cells transfected with the parental vector pSV2cat (control cells) with those synthesized in cells transfected with pSV2cat-DmH2a/b (experimental cells) indicated that the experimental cells contained higher concentrations of polyadenylate-minus

(poly(A⁻)) RNA that hybridized with isolated Drosophila H2a and H2b histone gene sequences than did control cells (Fig.2B; Fig.4B). Furthermore, most of this histone-specific RNA synthesized in the experimental cells is very rapidly lost from the CV-1 cells suggesting a rapid turnover or degradation of the insect histone messenger RNA in the cells (Fig.2B). This observation may partially explain the low levels of Drosophila histone proteins noted previously. Additionally, since apparently all of the hybridizable histone-specific messenger RNA (mRNA) synthesized in the transfected cells was in the poly(A⁻) RNA fraction (Fig. 2B; Fig. 4B), the results suggest that the Drosophila histone genes are operating primarily from their own endogenous DNA control sequences (see below). A further point to note in Fig. 2B is that the isolated insect histone H2a and H2b gene probes used in the hybridizations cross-reacted with the endogenous mammalian cell poly(A⁻) histone mRNA. This finding is to be expected given the fact that certain "domains" or regions of the H2a and H2b histone proteins and genes have been highly conserved during the evolution of most eukaryotic cells (13,37).

CV-1 Transfection with pRSV-DmH2a:

The efficiency of transcription of a gene is, among other things, a function of the type of promoter sequence controlling the activity of that gene. One of the most efficient promoter sequences so far analyzed in mammalian expressing vectors is contained in the long terminal repeat sequence of Rous sarcoma virus (11,18). When this LTR is placed adjacent to the bacterial CAT gene (replacing the 5' upstream SV40 early promoter in pSV2cat), the level of CAT enzyme activity is in most cell types, 3-5 times higher than that found in the same cells transfected with the parental pSV2cat vector (11). We therefore constructed a recombinant vector in which the Drosophila H2a gene was placed adjacent to the Rous LTR sequence to determine whether this promoter sequence would direct high levels of expression of insect histone RNA or protein in transfected cells.

Figure 3 shows the results of an experiment in which cells were transfected with either pRSV-DmH2a (experimentals) or with the parental vector pSV2cat (controls), labeled with (¹⁴C)-methionine, and the nuclear histones isolated and analyzed. The isolated histones from both experimental and control cells, along with marker histones isolated from Drosophila embryo cells and calf thymus tissue, were separated by electrophoresis on AUT-polyacrylamide gels (23,24). Panel A of this figure shows the Coomassie Blue stained protein profiles of the nuclear histones. Identifications of the various histone species are based on both the published literature (23-26) and on

Figure 3: Separation of Histones on Acid-Urea-Triton (AUT)-Containing Polyacrylamide Gels. Panel A: Coomassie Blue stained gel of separated histones. Lanes (1): *Drosophila* embryo marker histones; Lane (2): CV-1 cell histones labeled with (^{14}C)-methionine isolated from cells transfected with pSV2cat DNA (controls); Lane (3): CV-1 cell histones labeled with (^{14}C)-methionine isolated from cells transfected with pRSV-DmH2a plasmid DNA (experimental cells). In both the control and experimental cells isotopic labeling was started 24 hr post-transfection and continued for an additional 48 hr at 37°. The (^{14}C)-methionine concentration during labeling was 5 uCi/ml in methionine-free medium. Lane 4: Control calf thymus marker histones. Identification of the various histone protein species present on the gels is based on both published reports (23-26) and on unpublished control experiments. The major CV-1 cell H2a variants shown in lanes 2 and 3 are tentatively identified as H2a.1 and H2a.2 (25,26) and are labeled as .1 and .2. PANEL B: Fluorograph of the (^{14}C)-methionine labeled histone shown in Panel A. The CV-1 monkey kidney cell H2a histone variant labeled with methionine, tentatively identified as the H2a.2 variant, is designated as (.2) in the figure. The isotopically labeled histone band in the transfected cells which co-electrophoreses with authentic *Drosophila* embryo H2a histone (23) is labeled as such. Lane (2): Total nuclear histones from CV-1 cells transfected with the plasmid pSV2cat DNA (controls); Lane (3): Total nuclear histones isolated from CV-1 cells transfected with pRSV-DmH2a plasmid DNA (experimentals). PANEL C: Fluorograph of the intermediate lysine-rich histone fraction isolated from transfected cells labeled with (^{14}C)-methionine. The preferential extraction of the H2a and H2b histone fraction was based on the method of Ohlenbush et al.(41). Lane (1): Intermediate lysine-rich histone fraction from CV-1 cells transfected with calf thymus DNA (controls); Lane (2): Intermediate lysine-rich histone fraction from cells transfected with pRSV-DmH2a plasmid DNA. Protein labels as in the other panels.

unpublished control experiments. In particular, it is to be noted that even on these short one-dimensional AUT-polyacrylamide gels the authentic *Drosophila* H2a histone migrates slightly slower than the CV-1 H2a histone variant tenta-

tively identified as H2a.1 (25,26) and considerably slower than the monkey cell variant identified in the figure as H2a.2. When the CV-1 and Drosophila histones are separated by electrophoresis on longer AUT-gels the mobility difference between the insect and mammalian H2a histones is more obvious (for example, see Figs. 3C and 6A and reference 23).

Panel B of Figure 3 shows a fluorograph (28) of the same (^{14}C)-methionine labeled control and experimental cell histones shown in Panel A of the figure. Two points are to be noted: i) In the histones isolated from cells transfected with pRSV-DmH2a (lane 2), a methionine-labeled histone band is present that has a similar electrophoretic mobility as the authentic Drosophila embryo H2a histone; however, in the control, pSV2cat transfected cells, such a band is not found (lane 1); ii) Of the two major H2a histones (tentatively identified as H2a.1 and H2a.2; 25,26) found in CV-1 cells (cf., Panel A), only the H2a.2 variant is labeled with (^{14}C)-methionine and this variant is well separated on AUT-gels from the methionine-containing Drosophila H2a histone.

The electrophoretic profile of the methionine-labeled nuclear histones in CV-1 cells can be considerably simplified by preferentially extracting the group of intermediate lysine-rich proteins (mainly the H2a and H2b histones) from chromatin using the procedure of Ohlenbush et al. (27). When this is done on the labeled chromatin isolated from control and pRSV-DmH2a transfected experimental cells and the histones separated by electrophoresis on long AUT-gels, an unambiguous separation of the newly synthesized insect and mammalian histones is achieved as shown in the fluorograph in Panel C of Figure 3. Of course, the separation of insect and mammalian histones can also be unambiguously accomplished by two-dimensional polyacrylamide gel electrophoretic systems (22,25; unpublished observations) but in our hands the present system of selective histone extraction combined with one-dimensional electrophoresis is more convenient for screening.

Peptide Map of Drosophila H2a Histones:

Evidence that the radioactive protein band identified as Drosophila H2a histone in Figure 3 is probably authentic insect histone species is provided in Figure 4A. Authentic Drosophila H2a embryo histone has a molecular weight of approximately 14,500 daltons and contains a single internal methionine residue (16,23). From the known partial nucleotide sequence of this histone gene (16) the internal methionine residue is located at amino acid position number 54. Cyanogen bromide cleavage (29) of the molecule at this residue would be expected to release two peptide fragments with molecular weights of approximately 6,000 and 8,500 daltons. That this prediction is

Figure 4A: Comparison of the Cyanogen Bromide Cleavage Products of Authentic *Drosophila* H2a Embryo Histone and the Putative Insect H2a Histone Synthesized in Transfected CV-1 Cells. (^3H)-lysine labeled authentic *Drosophila* embryo H2a histone was isolated from isotopically labeled cells of the Schneider *Drosophila* tissue culture cell line followed by gel electrophoretic purification of the protein (46). The (^3H)-lysine labeled putative *Drosophila* H2a histone was similarly isolated from CV-1 cells transfected with plasmid DNA containing the *Drosophila* H2a histone gene. The purified proteins were then individually cleaved with cyanogen bromide. The resulting peptides were separated by electrophoresis on long 25% polyacrylamide gels containing sodium dodecylsulfate (46) and the radioactive bands located by fluorography. For comparisons, approximately equal amounts of radioactivity (i.e., cpm; in this instance, between 400-800 cpm each) were electrophoretically separated on the gels for the two different types of samples. Lane (1): Authentic H2a *Drosophila* histone; Lane (2): Putative *Drosophila* histone isolated from transfected CV-1 cells; Lane (3): Cyanogen bromide cleavage fragments of authentic *Drosophila* histone; Lane (4): Cyanogen bromide cleavage fragments of putative *Drosophila* histone isolated from CV-1 cells. The fluorographic exposure was for 13 weeks. **Fig.4B:** RNA "Dot-Blot" Assay of Hybridizable *Drosophila* H2a Histone Messenger RNA in Transfected CV-1 Cells. Total RNA was isolated from transfected cells and separated into poly(A⁻) and poly(A⁺) fractions. The various RNA preparations (5 ug of each) were spotted and covalently bonded to activated diazobenzyloxymethyl (DBM) paper and hybridized to an isolated *Drosophila* H2a gene fragment labeled at the 5' terminal ends with (^{32}P)-phosphate. An autoradiograph of the hybridization filter is shown in this panel. Columns are labeled according to the fraction of RNA originally spotted on the filter and the rows as follows: a) RNA isolated from CV-1 cells transfected with pSV2cat-DmH2a/b; b) RNA isolated from cells transfected with pRSV-DmH2a; c) Control CV-1 cells transfected with calf thymus DNA.

fulfilled when both authentic *Drosophila* H2a histone (isolated from *Drosophila* Schneider tissue culture cells labeled with (^3H)-lysine) and putative insect H2a histone (isolated from pRSV-DmH2a transfected cells and labeled with

Figure 5: Determination of the 5' Terminal Start Site for H2a Gene Transcription. Two determination methods were used; i) A modification (31) of the Berk-Sharp S1 nuclease mapping procedure (30) (results shown in Panel A); And, ii) An in vitro primer extension method using cytoplasmic RNA as the template and a histone gene-specific DNA fragment as a primer for elongation of a cDNA copy of the hybridized RNA by reverse transcriptase (32) (results shown in Panel B). The diagram in the lower part of the figure shows the region of pRSV-DmH2a vector DNA between the end of the LTR region and the 5' end of the *Drosophila* H2a histone gene. Diagram (a) shows the expected results of S1 nuclease protection experiments if the histone gene transcripts initiate synthesis at the known LTR "start" site (18) in the vector. In this experiment a 674 bp long Sph I/Ava I restriction fragment (—*) was 5' terminally labeled (*) with (32P)-phosphate and then restricted with Eco RI. The 589 bp long Eco RI/Ava I fragment (labeled at its 5' Ava I end with (32P)) was purified by agarose gel electrophoresis and then hybridized in excess to cytoplasmic RNA (~~~~) isolated from transfected CV-1 cells. S1 resistant hybrid molecules were denatured with glyoxyl and then separated by electrophoresis and the radioactive bands transferred to nitrocellulose filters (33) and detected by autoradiography (Panel A). In this experiment no radioactive band is present on the gel due to self-reannealing of the labeled DNA probe since the overhanging 5' Ava I end is susceptible to S1 nuclease cleavage in such hybrids (31). As shown in diagram (a), an approximately 537 bp long

S1 nuclease protected DNA fragment is expected if transcription begins at the Rous LTR "start" site whereas a much shorter 255 bp protected fragment is expected if the *Drosophila* 'cap sequence' is used for initiation. Panel A: Results of S1 nuclease protection experiment. Lane (1): Marker pBR322 Taq I DNA restriction fragments; Lane (2): S1 nuclease resistant fragment protected by cytoplasmic RNA from transfected CV-1 cells. Diagram (b) shows the *in vitro* primer extension results expected when a 234 bp long Hha I/Hha I restriction fragment (—) which overlaps the 5' end of the H2a gene is hybridized to cytoplasmic RNA (~~~~) and then extended with reverse transcriptase to give a radioactive cDNA copy (---) of the 5' end of the histone messenger RNA. A 438 bp long cDNA fragment would be expected if the histone mRNA began transcription at the LTR "start" site whereas no extended (radioactive) fragment would be expected if initiation begins at the 'cap' sequence. Lane (1): Marker pBR322 Taq I DNA restriction fragments; Lane (2): Extended cDNA fragment copied from cytoplasmic RNA isolated from CV-1 cells transfected with pRSV-DmH2a.

(³H)-lysine) are cleaved with cyanogen bromide is shown in Figure 4A. The molecular size markers shown in this figure are only approximate, but it is clear that both the authentic and the putative *Drosophila* H2a histones give essentially identical cleavage fragments strongly suggesting identity of the proteins. However, definitive proof that the putative protein is indeed the insect H2a histone requires further peptide analysis using a variety of different proteolytic enzymes since the methionine-containing CV-1 H2a histone might also give cyanogen bromide fragments (13) that are difficult to resolve from their insect counterparts on these low resolution gels. Nevertheless, from this and other accumulated results, we consider it highly likely that the cells transfected with pRSV-DmH2a are indeed synthesizing authentic *Drosophila* H2a histone proteins.

Comparison of pRSV-DmH2a and pSV2cat-DmH2a/b:

It is apparent that the plasmid pRSV-DmH2a is considerably more efficient than the construct pSV2cat-DmH2a/b in the production of insect histones in transfected cells. This also appears to be true for the insect histone messenger RNAs synthesized by these two different plasmids as shown by the results in Fig. 4B of an RNA 'dot-blot' hybridization assay (45). Although the results are only qualitative in nature, the autoradiograph in Fig. 4B suggests that pSV2cat-DmH2a/b induces considerably more hybridizable H2a histone mRNA in transfected cells than is found in mock-transfected control cells. The amount of hybridizable histone mRNA found in cells transfected with pRSV-DmH2a appears to be substantially higher. However, the main point to be noted in this figure is that, in contrast to the normal messenger RNAs found in both control and pSV2cat-DmH2a/b transfected cells, a substantial fraction of the histone mRNA found in pRSV-DmH2a transfected cells appears

to be polyadenylated at its 3' end since it binds selectively to affinity columns of oligo(dT)-cellulose (39). This finding is also supported by other data (unpublished observations).

Rous LTR Promotion of the H2a Histone Gene:

Figure 5 shows that the Rous LTR positioned upstream from the Drosophila H2a gene in the construct pRSV-DmH2a acts as a promoter for this gene. Two different procedures were used to identify the in vivo 5' end of the H2a mRNA in transfected cells. The first was a modification of the Berk-Sharp S1 nuclease RNA mapping procedure (30,31) and the second was an in vitro primer extension method using a gene-specific DNA fragment as primer and cytoplasmic RNA as template (32).

For the S1 nuclease mapping experiments, total cellular RNA from pRSV-DmH2a transfected cells was isolated and hybridized to a 5' (32P)-end labeled Eco RI/Ava I restriction fragment from pRSV-DmH2a. As shown in the lower diagram in Fig. 5, this labeled DNA fragment (*) overlapped both the 5' end of the Drosophila H2a gene and part of the upstream Rous LTR sequence. After S1 nuclease digestion, resistant hybrid molecules were denatured with glyoxal and electrophoresed on an alkaline 1.8% agarose gel, the separated DNA fragments transferred to a nitrocellulose filter by the method of Southern (47), and the radioactive bands detected by autoradiography.

Although the DNA sequence coding for the actual 5' terminus of the Drosophila H2a histone mRNA is not known, it is likely that it, like the sea urchin H2a histone (34-37) and several other histone genes (35,37), is in, or adjacent to, the so called "cap sequence" (5'pyCATTCPu3') upstream from the AUG initiation codon in the histone gene. If this "cap sequence" is the in vivo start site for the insect H2a gene in transfected cells then in the S1 nuclease protection experiments a radioactive DNA fragment about 255 nucleotides in length should be obtained (Fig. 5 and reference 29). However, as seen in Figure 5 (Panel A, lane 2) the actual size of the major RNA-protected radioactive DNA fragment is greater than 475 nucleotides in length (c.f., lane 1, Panel A). These results therefore show that the start site for the mRNA lies farther upstream in the Rous LTR region whose known promoter start site (31) is about 537 nucleotides upstream from the Ava I site in the H2a gene (Fig.5, diagram a).

Further evidence suggesting that the Rous LTR sequence is the start site for H2a mRNA synthesis comes from the results of in vitro primer extension studies shown in Figure 5B. In these experiments a 234 bp long Hha I restriction fragment which overlaps both the 5' end of the H2a gene and the upstream

sequences which include the "cap sequence" and the "TATA" box (Fig.5, diagram b) was isolated and hybridized to total cytoplasmic RNA isolated from transfected cells. Avian myeloblastosis virus reverse transcriptase enzyme was used to make a radioactive cDNA copy of the hybridized mRNA molecules extending to near the 5' end of the messages. When the products of this reaction were analyzed as above, a single radioactive DNA band greater than 368 bp, but less than 475 bp, in length was observed (Fig.5B). A radioactive fragment approximately 438 nucleotides in length would be expected if the histone mRNA began transcription at the Rous LTR start site. Conversely, no detectable fragment at all would be expected if the mRNA had initiated synthesis at the insect gene "cap sequence" (Fig. 5, diagram b). Therefore, taken together, these results suggest that the 5' end of most of the hybrid Drosophila histone mRNA is within the Rous LTR region and that the transcript includes both the truncated end of the H2b histone gene and the spacer region between the insect genes. When similar experiments are performed with RNA isolated from control CV-1 cells no such histone mRNA transcripts are found (data not shown).

Drosophila H2a Histone Associated With Monomer Nucleosomes:

We next asked whether the Drosophila H2a histones synthesized from the pRSV-DmH2a template in transfected monkey cells are functional, at least in the sense that they could be incorporated into mammalian cell chromatin. Twenty-four hours after transfection of CV-1 cells with pRSV-DmH2a, (^{14}C)-methionine was added to the cells and labeling continued for another 62 hours. Nuclei (about $5 \times 10^7/\text{ml}$) were isolated from the cells and digested with micrococcal nuclease (200 units/ml, 10 min, 37°) to release monomer nucleosomes (38). The released chromatin particles were separated by centrifugation on a 10-30% sucrose density gradient; fractions were taken and monitored for absorbance at 260 nm (Fig. 6A). The optical density peak corresponding to the monomer nucleosome fraction (Fig. 6B) was taken and examined for the presence of labeled Drosophila H2a protein. The H2a and H2b histones were preferentially extracted (27) from the monomer peak (panel A) and the proteins separated by electrophoresis on an AUT gel (panel A, insert). From this figure, it is evidence that cells transfected with pRSV-DmH2a, but not control cells, contain in their nucleosomes labeled histone proteins co-migrating with authentic Drosophila H2a proteins (Fig. 6A). Additionally, control experiments in which free, radioactively labeled, Drosophila histones were added to the preparations of CV-1 nucleosomes prior to their separation by centrifugation on sucrose gradients, demonstrated that the insect histones do not become adventitiously bound to the control CV-1 monomer nucleosomes (Fig. 6A).

Figure 6: Detection of *Drosophila* H2a Histone in Mammalian Nucleosomes.

Micrococcal nuclease digestion was used to release nucleosomes from both control CV-1 cells and CV-1 cells transfected with pRSV-DmH2a which had been labeled with (^{14}C)-methionine after DNA transfection. The released chromatin particles were separated on a 10-30% sucrose density gradient by centrifugation and fractions of the gradient collected and the absorbance at 260 nm determined (—). The H2a and H2b histone fractions were preferentially extracted (27) from the monomer nucleosome peak (Panel A), the proteins separated on an AUT-gel (c.f. Fig.3), and the radioactive bands detected by fluorography (Panel A, Insert). Lane (1) of the insert contains the labeled histones from control CV-1 cells transfected with pSV2cat plasmid DNA; Lane (2) of the insert contains labeled histones isolated from pRSV-DmH2a transfected CV-1 cells. The labels in this insert are the same as in Fig.3. In control experiments, "free" (3H)-lysine labeled *Drosophila* histones were added to CV-1 nucleosome preparations prior to gradient centrifugation and the distribution of the radioactive label subsequently monitored (●—●) in the gradient after centrifugation. As can be seen in Panel A, the "free" histone remains at the top of the sucrose gradient. Panel B: Pattern obtained when the DNA fragments isolated from the monomer nucleosome peak in Panel A were electrophoresed on a 1.4% agarose gel and then visualized under ultraviolet light after staining with ethidium bromide (Lane 1). It can be seen that the DNA fragments isolated from the monomer nucleosome peak co-migrate with authentic monomer sized DNA fragment (M) electrophoresed on a parallel gel (Lane 2). Labels: M, monomer nucleosome size DNA; D, dimer nucleosome size DNA; T, trimer nucleosome size DNA.

These results therefore strongly suggest that the insect H2a histone has actually been incorporated into the mammalian nucleosome structure. This interpretation is further supported by the fact that any loosely bound proteins would likely have been removed from the isolated nucleosomes by the high salt washing procedure employed prior to the selective extraction of the intermediate lysine rich histones (27).

DISCUSSION

In agreement with previous reports (1-5), the findings presented here demonstrate that the position of eukaryote vector control sequences can affect the function of foreign genes inserted into such vectors. For example, when a DNA fragment containing both the H2a and H2b histone genes of Drosophila (along with their endogenous controlling sequences) is placed in the unique Bam HI site of the pSV2cat vector (Fig. 1), the efficiency of expression of the insect genes is low (Fig. 2B; Fig. 4B). When the insect genes are in this position in the vector their expression most likely is controlled by the insect promoter and terminator DNA sequences although the level of their expression could also be affected by the SV40 enhancer sequences present on the plasmids (19,47). Evidence supporting this interpretation comes from the observation that the histone messengers transcribed from the insect genes in this construction lack a 3' polyadenylate tail sequence (Fig. 2B; Fig. 4B). Additionally, we have found that the isolated Bam HI DNA fragment containing the insect histone genes will itself give low efficiency histone gene expression when used in DNA transfection experiments (unpublished data), again suggesting that the insect histone genes are functioning primarily from their own control sequences. Similar conclusions about the Drosophila histone genes have been arrived at by Mertz (21,22) from experiments in which the insect genes have been microinjected into Xenopus oocytes. Furthermore, the rapid turnover of the poly(A⁻) histone mRNA within transfected cells (Fig. 2B) is reminiscent of the types of controls known to affect the level of functional histone mRNA in both Drosophila embryos (40) and yeast cells containing extra copies of the histone genes (41).

A very different situation exists when the vector pRSV-DmH2a is used to introduce Drosophila histone genes into cells. In this case, transfected cells show readily detectable levels of both insect histone mRNA and protein (Figs. 3-6). Furthermore, the structure of the majority of the insect histone mRNAs found in the transfected cells is different from that normally found in Drosophila cells (37,40). For example, instead of initiating at the

expected upstream 5' histone "cap sequence" (35,37), the bulk of the mRNA appears to begin transcription farther upstream somewhere in the Rous long terminal repeat sequence (Fig. 5). This finding is similar to a situation reported by Gorman et al. (11) in which transcription of the bacterial CAT gene, placed in the same vector position as the histone gene in the present construct, initiates at the Rous LTR 'start' site.

It also appears that a substantial fraction of the histone mRNA transcribed from the pRSV-DmH2a vector, in contrast to most Drosophila histone mRNAs, has a 3' poly(A⁺) tail (Fig. 4B). This is most likely the result of the gene having been placed (see Fig. 1) adjacent to the SV40 small tumor antigen intron/exon splice site and upstream from the SV40 early region polyadenylation site (1,3). Recently, Canaani and Berg (5) have reported that transcripts from the bacterial neomycin resistance gene "neo", which encodes for an aminoglycoside phosphotransferase and whose messenger RNA normally lacks a 3' polyadenylate tail, contains a 3' poly(A⁺) tail when the gene is placed adjacent to the SV40 tumor antigen 3' processing sequences of pSV2 vectors. Similarly, in unpublished experiments (mentioned in references 1 and 2), Hofstetter and Berg have placed the Drosophila H2b gene sequence in the "late" transcript region of SV40 viral constructs and have apparently observed the formation of both a cytoplasmic, polyadenylated SV40-DmH2b hybrid histone mRNA and H2b protein in transfected monkey tissue culture cells. Together, all of these results suggest that in the vector pRSV-DmH2a the normal insect histone gene initiation and termination signals (which are still present) have, for the most part, been "superseded" in activity by the the adjacent viral DNA regulatory sequences present in the vector.

Experimental evidence reported by other workers has suggested that the addition of a 3' polyadenylate tail to a normally deficient histone mRNA molecule will result in marked stabilization of this mRNA when it is micro-injected into Xenopus oocytes(44). It has also been suggested that during oogenesis and early embryogenesis of Xenopus the polyadenylation of histone mRNA might be an important control mechanism for the regulation of histone gene expression in this species (42,43). When this information is combined with the observation that the Rous sarcoma virus LTR has been demonstrated to be an efficient promoter of downstream coding sequence (11), it becomes more reasonable to accept the relatively high efficiency of expression of the Drosophila histone genes in mammalian cells transfected with the vector pRSV-DmH2a. However, regardless of its cause, this increased efficiency has allowed us to demonstrate the presence of insect H2a histone proteins in

isolated monomer nucleosomes of CV-1 monkey cells (Fig. 6). Since control experiments show that free Drosophila histones do not become adventitiously associated with monomer nucleosomes isolated from mammalian cells, we think it highly likely that the insect histones have actually become part of the nucleosome structure of the transfected cells. Such a situation, where the histones from different phylogenetic classes (insects and mammals) can interact to form bona fide chromatin subunits, should come as no surprise considering the highly conserved nature of histones in evolution (13,37) and given the fact that even histones derived from different phylogenetic kingdoms (e.g., plants and animals) can interact with each other in apparently normal ways in vitro (13).

The ability, which we have demonstrated here, to change the protein composition of mammalian cell chromosomes by the introduction of foreign genes into cells may allow for the in vivo 'remodeling' of chromatin in precisely defined ways. Furthermore, the techniques involved may be generally applicable for use with other genes coding for chromosomal proteins.

ACKNOWLEDGMENTS

This work was supported in part by N.I.H. grant # GM26702 (to R.R.) and by a fellowship from the American Cancer Society (# PF1926 to C.M.G.). We would like to thank Dr. George Spiegelman (Univ. of British Columbia) for supplying us with the cDm500 plasmid and Dr. Ira Pastan (N.I.H.) for allowing us to use the Rous LTR sequence. Special thanks also are owed to Dr. Michael Kahn and Dr. Michael Smerdon (Washington State University) for invaluable assistance and useful discussions during the course of this work.

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