c-Ha-*ras* $^{Val\ 12}$ Oncogene-transformed NIH-3T3 Fibroblasts Display More Decondensed Nucleosomal Organization than Normal Fibroblasts

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Abstract. We have compared the nucleosomal organization of c-Ha-*ras* $^{Val\ 12}$ oncogene-transformed NIH-3T3 fibroblasts with that of normal fibroblasts by using micrococcal nuclease (MNase) as a probe for the chromatin structure. The bulk chromatin from asynchronously and exponentially growing *ras*-transformed cells was much more sensitive to MNase digestion than chromatin from the normal cells. Southern hybridization analyses of the MNase digests with probes specific for the *ornithine decarboxylase* (*odc*) and c-*myc* genes showed that the coding and/or 3′ end regions of these growth-inducible genes carry a nucleosomal organization both in *ras*-transformed and normal cells. Studies with cells synchronized by serum starvation showed that in both cell lines the nucleosomal organization of chromatin is relatively condensed at the quiescent state, becomes highly decondensed during the late G_1 phase of the cell cycle, and starts again to condense during the S phase. However, in *ras*-transformed cells the decondensation state stayed much longer than in normal cells. Moreover, irrespective of the phase of the cell cycle the bulk chromatin as well as that of the *odc* and c-*myc* genes was more sensitive to MNase digestion in the *ras*-transformed cell than in the normal fibroblast. Decondensation of the chromatin was also observed in the normal c-Ha-*ras* protooncogene-transfected cells, but to a lesser extent than in the mutant *ras*-transformed cells. Whether the increased degree of chromatin decondensation plays a regulatory role in the increased expression of many growth-related genes in the *ras*-transformed cells remains an interesting object of further study.

THE chromatin of higher eukaryotes consists of chromatin subunits, in which 146 bp of DNA are wrapped around a histone octamer forming the core nucleosome. This contains two each of the histones H2A, H2B, H3, and H4 (Igo-Kemenes et al., 1982). Association of a fifth histone, H1, to the core particle or to the linker DNA (Igo-Kemenes et al., 1982; Weintraub, 1984; Widom and Klug, 1985; Felsenfeld and McGhee, 1986) is a requisite for condensation of the 10-nm nucleosome fiber ("beads-on-a-string" form) into the 30-nm fiber (Igo-Kemenes et al., 1982; Widom and Klug, 1985; Felsenfeld and McGhee, 1986).

Micrococcal nuclease (MNase)[1] is a highly informative probe for the organization of chromatin digesting DNA between two adjacent nucleosomes (Finch et al., 1975). MNase is relatively insensitive to foreign ions or molecules (Prentice and Gurley, 1983) and digests almost equally well isolated nucleosomes, isolated chromatin, or chromatin of the cell nucleus (Axel, 1978). It produces a ladder of several DNA bands on gels as visualized by ethidium bromide staining. These digestion patterns vary greatly depending on the level of chromatin condensation. The binding of certain histone H1 variants appears to be associated with chromatin condensation and repression of gene expression (Weintraub, 1984; Allan et al., 1980; Cole et al., 1977; Roche et al., 1985; Bergman et al., 1988). Stripping off the histone H1 variant H1° or H5, an avian homologue of the mammalian H1°, from isolated nuclei (Cole et al., 1977) or purified chromatin (Allan et al., 1980) renders the chromatin more sensitive to MNase digestion. Addition of exogenous H5 reverts this effect (Allan et al., 1980). Furthermore, it is known that MNase digests more rapidly decondensed chromatin of the interphase nucleus than condensed chromatin of the mitotic nucleus (Axel, 1978).

Increased sensitivity to MNase or DNase I correlates with transcriptional activation of genes (Eissenberg et al., 1985). It is believed that most transcribed genes carry a nucleosomal organization (Chou et al., 1986; Chen and Allfrey, 1987; Pavlovic et al., 1989), although this may be lacking in highly expressed genes as well as in the 5′ regions of many genes (Pavlovic et al., 1989; Wu et al., 1979; Moreno et al., 1986). For example, Stein and co-workers (Moreno et al., 1986) showed recently that the 5′ region of the *H4* gene displays a disrupted nucleosomal organization in the S phase when the gene is actively transcribed, while the 3′ coding region displays nucleosomal organization throughout the whole cell cycle.

Cell transformation is known to be associated with aberrant expression of many genes. However, very little information is available on the potential changes in chromatin structure of these genes or bulk chromatin in the transformed

1. *Abbreviation used in this paper*: MNase, micrococcal nuclease.

cells. We have previously reported that c-Ha-*ras* $^{Val\ 12}$ oncogene-transformed mouse NIH-3T3 fibroblasts (E4) display an increased expression of the *ornithine decarboxylase (odc), glucose transporter, transin*, and *jun* genes as compared to the normal fibroblasts (Sistonen et al., 1987; Hölttä et al., 1988; Sistonen et al., 1989). Here we addressed the question of whether there are any differences in the organization of chromatin between the normal and *ras*-transformed cells.

Materials and Methods

Cells

The NIH-3T3 cells transfected with the pSV2 *neo* plasmid alone (N1, Sistonen et al., 1987) and those cotransfected with the c-Ha-*ras* $^{Val\ 12}$ oncogene (E4, Sistonen et al., 1987) or c-Ha-*ras* protooncogene (B8, Hölttä et al., 1988) were routinely grown on plastic petri dishes (diameter 9 cm), in DME containing 10% FCS supplemented with penicillin (125 U/ml) and streptomycin (125 μg/ml).

For synchronization, the cells were grown to semiconfluency in ordinary medium. The cell cultures were then incubated for 24 h in DME without serum. The quiescent cells were allowed to reenter the growth cycle by addition of DME containing 10% dialyzed FCS.

Cytological Stainings

The determination of mitotic index was carried out by counting the number of mitoses from at least 400 cells using preparations fixed either with ethanol or methanol and stained with May-Grünwald-Giemsa or Hoechst 33258 fluorochrome (2.5 μg/ml), respectively.

Cell Cycle Analysis by Flow Cytometry

The DNA content of the cell nuclei stained with ethidium bromide was analyzed by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems, Mountain View, CA).

^{3}H-Methylthymidine Incorporation

For determination of the cell cycle phases following synchronization, 8 μCi ^{3}H-methylthymidine was added to the cultures (~40,000 cells/cm^2) for 30 min at 2-h intervals after serum addition. The cells were then washed twice with ice-cold PBS and detached using a rubber policeman. Cells were collected by centrifugation and extracted twice with 1 ml of ice-cold 5% TCA. The final TCA-insoluble pellet was dissolved in 0.1 M NaOH and counted in ACS squintillation liquid (Amersham International, Amersham, UK) in a Rack-Beta counter (LKB-Wallac, Finland).

Determination of Ornithine Decarboxylase Activity

For measurement of the activity of ornithine decarboxylase, the cells on the culture substratum were washed twice with ice-cold PBS and detached with a rubber policeman. The cells were harvested by centrifugation, resuspended in a buffer containing 25 mM Tris; pH 7.5, 0.1 mM EDTA, 2 mM DTT, and disrupted by freezing and thawing twice. Determination of the ornithine decarboxylase activity in the high-speed supernatant fraction was carried out as described earlier (Sistonen et al., 1987, Hölttä et al., 1988).

Isolation of Nuclei

Nuclei were prepared from 2–5 × 10^7 cells. Isolation of nuclei was carried out essentially as described by Axel (1978) and Moreno et al. (1986) with minor modifications. Before suspending the cells in RSB buffer (10 mM Tris-HCl; pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) two protease inhibitors, PMSF and N-ethylmaleimide, at final concentrations of 0.1 and 0.3 mM, respectively, were added to the RSB buffer. The suspended cells (10 × 10^6 cells/ml) were lysed by stepwise addition of 10% NP-40 to a final concentration of 0.5% and gentle vortexing (~10 s). Detergent extraction was repeated once. Nuclei were then centrifuged at 200 *g* for 5 min and washed three times with RSB buffer without the detergent. Intact nuclei without cytoplasmic remnants, as revealed by phase-contrast microscopy, were thus obtained. Centrifugation at the low centrifugal force enabled a homogenous resuspension of the nuclei. All steps were carried at temperatures between 0° and 4°C.

Small aliquots of the nuclear suspension were removed and diluted to 1/10 in RSB for counting, or diluted in 5.5 M urea and 2.2 M NaCl for determining the OD at the wavelength 260 nm. Calculations were made by assuming that $OD_{\lambda = 260\ nm} = 1$ corresponds to 50 μg/ml DNA. The calculated mass of DNA [μg]/million nuclei was found to correlate well with the data from cell cycle analysis with flow cytometry. Nuclei representing different stages of the cell cycle were stored at $OD_{\lambda=260\ nm} = 80$ in liquid N_2 in 40% glycerol before the micrococcal nuclease digestions. The storage of nuclei did not alter the digestion kinetics of the nuclease as revealed by a comparison to nuclei used immediately.

MNase Digestion

The MNase digestion was carried out as described elsewhere (Axel, 1978), except that a RSB buffer containing 0.1 mM $CaCl_2$ was used as the reaction buffer. The isolated nuclei were resuspended to a density of $OD_{\lambda = 260\ nm} = 10$, and the enzyme was added to a final concentration of 50 U/ml (for additional details see the Results section). After different times of incubation, aliquots (125 μl) of the digestion mixture (at 37°C) were removed and mixed with an equal volume of ice-cold 10 mM EDTA (pH 6.0) to stop the reaction. To check possible autodigestion, nuclei were resuspended in the reaction buffer and incubated without exogenous MNase at 37°C for 30 min. Also, nuclei were incubated in the presence of Ca^{2+}-ions at 4°C instead of 37°C for up to 12 h. Furthermore, nuclear preparations were analyzed for the possible endogenous DNase requiring Mg^{2+}-ions by incubating the nuclei without Ca^{2+} at 37°C for 30 min.

Isolation of DNA, Electrophoresis, and Blotting

DNA extractions were carried out as described elsewhere (Rill et al., 1978; Maniatis et al., 1984). Before the ethanol precipitations $MgCl_2$ was added to a final concentration of 10 mM to rescue small DNA fragments (Maniatis et al., 1984) present in the mononucleosomes. The nucleosomal organization of DNA from the digested nuclei was analyzed by electrophoresis in 1.6% agarose gels (5–8 μg DNA) followed by visualization with ethidium bromide staining and UV light. Fragment sizes were calculated using λ-phage DNA digested with Eco RI and Hind III (Boehringer Mannheim Biochemicals, W. Germany) as a marker. The degree of chromatin digestion was quantitated by densitometric scanning (EDC; Helena Laboratories, Beaumont, TX) of the photographs (negatives) of the digestion patterns. The DNA fragments were transferred by capillary blotting to Hybond-N nylon filters (Amersham International) in 6× SSC (1× SSC consists of 0.15 M NaCl and 0.015 M trisodium citrate) overnight. Thereafter, filters were dried and baked at 80°C for 1 h.

Hybridization Analyses

For detecting *odc*, *c-myc*, and *glyceraldehyde-3-phosphate dehydrogenase* genes, the filters were hybridized to the following nick-translated plasmid probes: pODC16 (Hickok et al., 1986), pSV-c-*myc* -1 (Land et al., 1983), and pRGAPDH-13 (Fort et al., 1985), respectively. pODC16 carries the mid-coding region as well as the 3'-nontranslated region of *odc* cDNA, the c-*myc* probe carries the second and third exons and an intron (Land et al., 1983), and the *gapdh* probe is a full-length cDNA. Standard nick-translation reactions gave specific activities of at least 10^8 cpm/μg DNA. Hybridizations were performed in a mixture of 5× Denhardt's solution (0.02% each of BSA, Ficoll, and polyvinyl-pyrrolidone), 5× SSPE, 50% formamide, 0.5% SDS, and herring sperm DNA (100 μg/ml) for overnight at 42°C. Filters were washed in 2× SSC, 0.1% SDS three times at room temperature for 5 min; in 0.3× SSC, 0.1% SDS at 42°C for 20 min; and finally in 0.1× SSC, 0.1% SDS at 42°C for 20 min. The filters were exposed to XAR - 5 films (Eastman Kodak Co., Rochester, NY) at −70°C for different times (3–10 d). For reprobing, the previous probe was removed by boiling the filters in 0.1% SDS for 5 min.

Results

Effect of Serum Stimulation on the Nucleosomal Organization of Bulk Chromatin from Normal NIH 3T3 Fibroblasts

To study how the chromatin structure of normal cells is

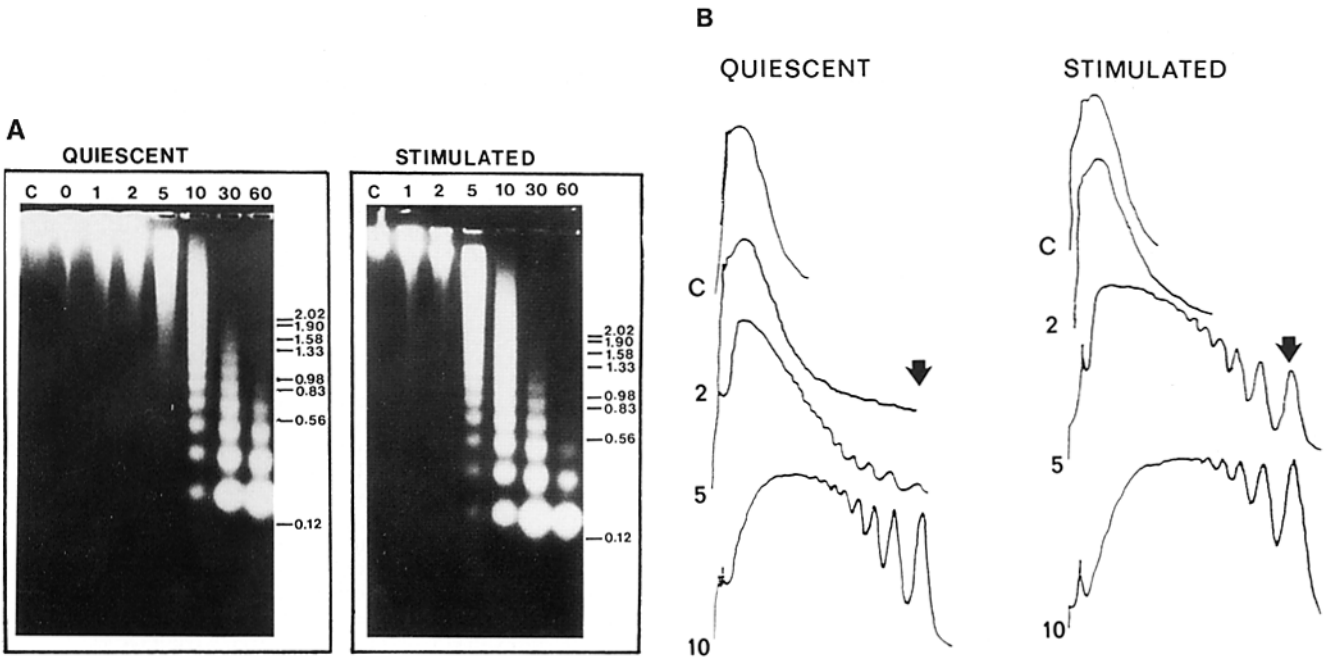

Figure 1. (*A*) MNase digestion of nuclei from quiescent and stimulated NIH-3T3 (N1) cells. Cells were stimulated by addition of 10% dialyzed FCS or made quiescent by serum starvation for 16 h, whereafter the nuclei were isolated and subjected to MNase digestion (50 U/ml) for the indicated times. The nuclei were kept at 4°C before adding MNase and shift to 37°C. DNA extracted from the digested nuclei was analyzed on 1.6% agarose gels and visualized by ethidium bromide staining and with UV illumination. The numbers on top indicate digestion time with micrococcal nuclease in minutes. *C* indicates DNA from undigested nuclei. Molecular weight markers (λ DNA cut with Eco RI and Hind III) are indicated on the right sides of the figures. (*B*) Densitometric scans of the electrophoretic patterns of the MNase digested DNA in *A*. The photographs (negatives) of the digestion patterns in *A* were scanned with a densitometer coupled to a computer. The arrows indicate the positions of DNA from the nucleosome monomer. The numbers on the sides indicate times of digestion. Migration is from left to right.

affected by the proliferation state of the cells, part of the subconfluent cultures of N1 fibroblasts were either stimulated by addition of dFCS or made quiescent by serum starvation for 16 h, whereafter the nuclei were isolated and subjected to MNase digestion. Fig. 1 *A* shows the nucleosomal organization of DNA from the digested nuclei analyzed by agarose gel electrophoresis and ethidium bromide staining. The bulk chromatin from both the quiescent and stimulated cells displayed a typical nucleosome ladder, which was, however, obtained more rapidly in the stimulated cells than quiescent cells after the MNase digestion (Fig. 1). The differences in the digestion kinetics are evident from the densitometric scans (Fig. 1 *B*). A mononucleosome versus oligonucleosome/DNA ratio calculated from the gels is significantly higher in the stimulated nucleus than in the quiescent nucleus. A 5-min digestion of the chromatin from the stimulated cell resulted roughly in a similar degree of degradation of the chromatin into the nucleosomes as a 10-min digestion of the quiescent cell nuclei (Fig. 1 *B*). These data indicate that the nuclei from exponentially growing (asynchronous) cells display a more decondensed nucleosomal organization than the nuclei of the resting cells (at G_0).

The Nucleosomal Organization of Bulk Chromatin from c-Ha-ras Oncogene-Transformed Cells

Next we studied whether there are differences in the nucleosome organization of chromatin between the normal and c-Ha-*ras* $^{Val\ 12}$ oncogene-transformed E4 fibroblasts. After stimulation with serum, nuclei of the exponentially growing E4 and N1 cells were isolated, digested with MNase, and resulting DNA fragments analyzed on agarose gels. As shown by densitometric scanning, bulk chromatin from the E4 cells shows a nucleosomal organization which is significantly more sensitive to MNase than that of the N1 cells. For example, 1 min digestion of the nuclei from the E4 cells shows almost the same degree of degradation as 10 min digestion of the N1 cell nuclei (Fig. 2). That the differences in the nucleosomal organization between the transformed E4 cells and normal N1 cells were not due to different rates of autodigestion catalyzed by endogenous nucleases was checked by incubating the nuclei in the presence of Ca^{2+}-ions without exogenous MNase at 37°C for 30 min. Fig. 2 shows that there is no significant autodigestion in the nuclei either from N1 or E4 cells (*AUTO* lane).

Chromatin Structure of the odc and c-myc Genes

In both N1 and E4 cells growing exponentially the *odc* gene displayed a nucleosomal ladder in its coding and 3′ region. The densitometric scanning results show that the *odc* gene, like the bulk chromatin of the E4 cells, has a significantly more MNase-sensitive nucleosomal structure than *odc* of the N1 cells (Fig. 2). The same is also true for the c-*myc* gene when analyzed by a probe hybridizing to the major coding exons II and III of c-*myc* (Fig. 2), as well as for the *glyceraldehyde-3-phosphate dehydrogenase (gapdh)* gene (Fig. 2).

As both N1 and E4 cell lines were transfected with the

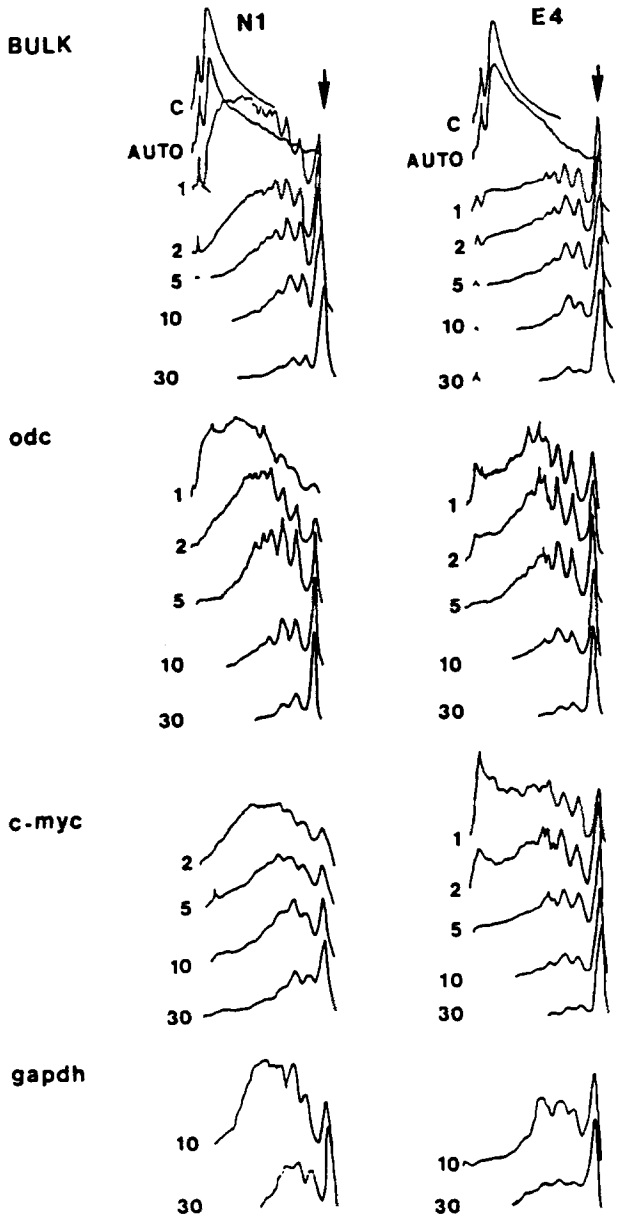

Figure 2. Densitometric scans of the electrophoretic patterns for the bulk chromatin and *odc* and c-*myc* genes from the nuclei of normal (*N1*) and *ras*-transformed (*E4*) cells digested with MNase. After stimulation with serum, nuclei of the exponentially growing E4 and N1 cells were isolated, digested with MNase, and the DNA fragments analyzed on agarose gels. The scans were either generated from photographs (negatives) or autoradiograms of the Southern blots hybridized with nick-translated probes specific to *odc*, c-*myc*, and *gapdh* genes. The arrows indicate the positions of DNA from the nucleosome monomer. The incubation times are indicated as in Fig. 1 *B*. *AUTO* refers to autodigestion.

pBR322 plasmid containing the *neomycin phosphotransferase* gene (*neo*) used for selection (Sistonen et al., 1987), we looked for eventual interference of the pBR322-sequences with the hybridization results. However, a nick-translated pBR322 plasmid did not hybridize to any appreciable extent with the nucleosomal ladder (data not shown).

Synchronization of Cells

Next, we wanted to compare the chromatin structure of the N1 and E4 cells at the different phases of cell cycle. For this we synchronized subconfluent N1 and E4 cells by serum starvation for 24 h. A highly synchronous population of starting cells was thus obtained. ^{3}H-methylthymidine incorporation studies indicated that the cells were arrested in G_0/G_1 phase (Fig. 3) and flow cytometrical analysis showed that 93% of the N1 cells and 90% of the E4 cells were blocked in G_1 (data not shown, see Fig. 6 *B*). Cells were then replated in fresh medium containing 10% dFCS to allow the cells to enter the growth cycle. The progress through the cell cycle was monitored (*a*) by measuring the ability of the cells to incorporate ^{3}H-methylthymidine into DNA; (*b*) by quantitating the DNA/nucleus ratio or by flow cytometry; and (*c*) by calculating the mitotic index after staining of the cells either with the Hoechst 33258 fluorochrome or May Grünwald Giemsa. The thymidine incorporation data in four separate experiments showed that the quiescent N1 fibroblasts entered the S phase 16 h after the addition of dFCS. The S phase lasted for ~12 h and the first mitotic cells (with a mitotic index of 0.10) appeared at 30 h. The DNA/nucleus ratio correlated well, although not strictly to the thymidine incorporation curve (*gray bars* in Fig. 3). A similar pattern of changes in the cell cycle parameters to that in N1 cells was also seen in E4 cells during the course of serum stimulation (Figs. 3 and 5), although the E4 cells appeared to reach the S phase slightly earlier than the N1 cells as determined by ^{3}H-methylthymidine incorporation (Fig. 3). In addition, analysis of the cellular DNA content by flow cytometry indicated that the N1 and E4 cells progressed through the cell cycle in a synchronous manner (data not shown). It can also be seen that the DNA/nucleus ratios of the exponentially

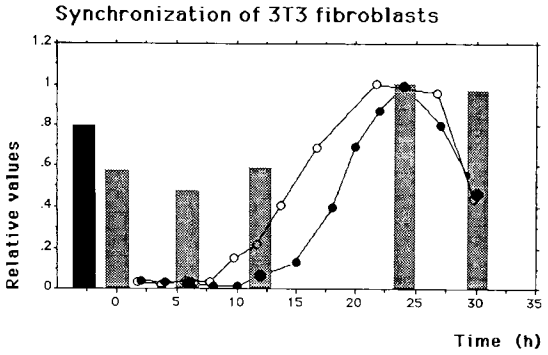

Figure 3. Synchronization of the normal N1 and *ras*-transformed E4 fibroblasts. N1 and E4 cells were synchronized by serum starvation for 24 h, whereafter serum was added to allow the cells to enter the cell cycle. DNA synthesis was determined by addition of 8 μCi ^{3}H-methylthymidine at 2-h intervals after serum addition and measuring the incorporation of radioactivity into the acid-insoluble fraction. The filled and open circles indicate the thymidine incorporation of N1 and E4 cells, respectively. For N1 cells the relative mass of DNA/million nuclei in the nuclear preparations is shown by bars. The black bar corresponds to the relative DNA/nucleus ratio of exponentially growing asynchronous cells. The x-axis indicates the time in hours after addition of serum to the serum-starved cells. The y-axis indicates relative values for the ^{3}H-methylthymidine incorporation into DNA and the DNA/nucleus ratios. The values are means from two to three independent experiments.

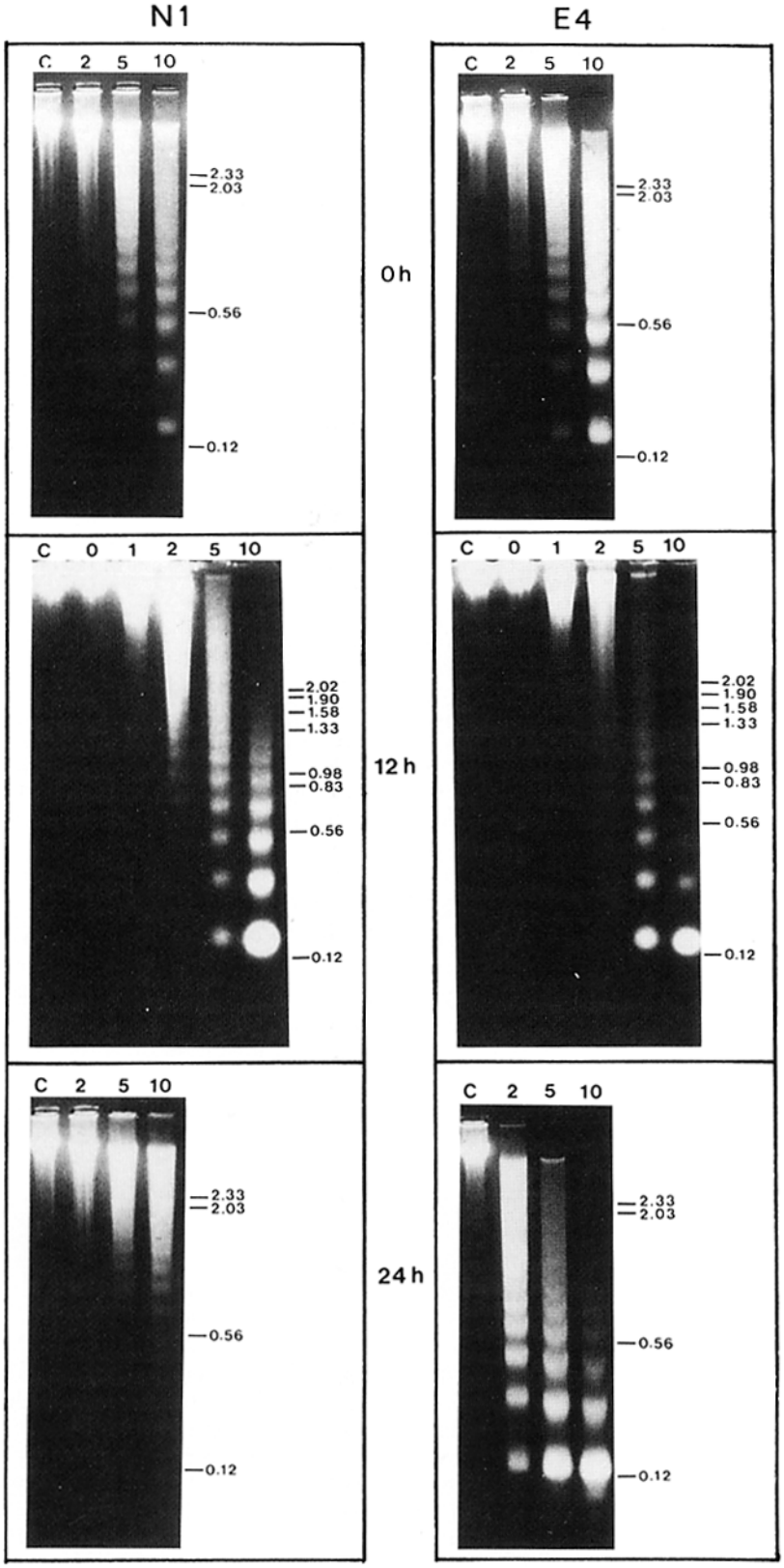

Figure 4. Nucleosomal organization of bulk chromatin during the cell cycle. After serum starvation, nuclei of synchronized E4 and N1 cells were isolated 0, 12, and 24 h after serum stimulation, digested with micrococcal nuclease and analyzed as in Fig. 1 *A*.

growing nonsynchronized N1 or E4 cells shown for comparison (*black bar* in Fig. 3) are somewhat smaller than those of the S phase cells.

We were also interested in measuring the activity of ODC, since it has been reported to show cell cycle-related fluctuations (Heby, 1981), and to see whether it correlates with the changes in the *odc* chromatin structure (see below). The activity of ODC displayed a threephasic increase in N1 cells

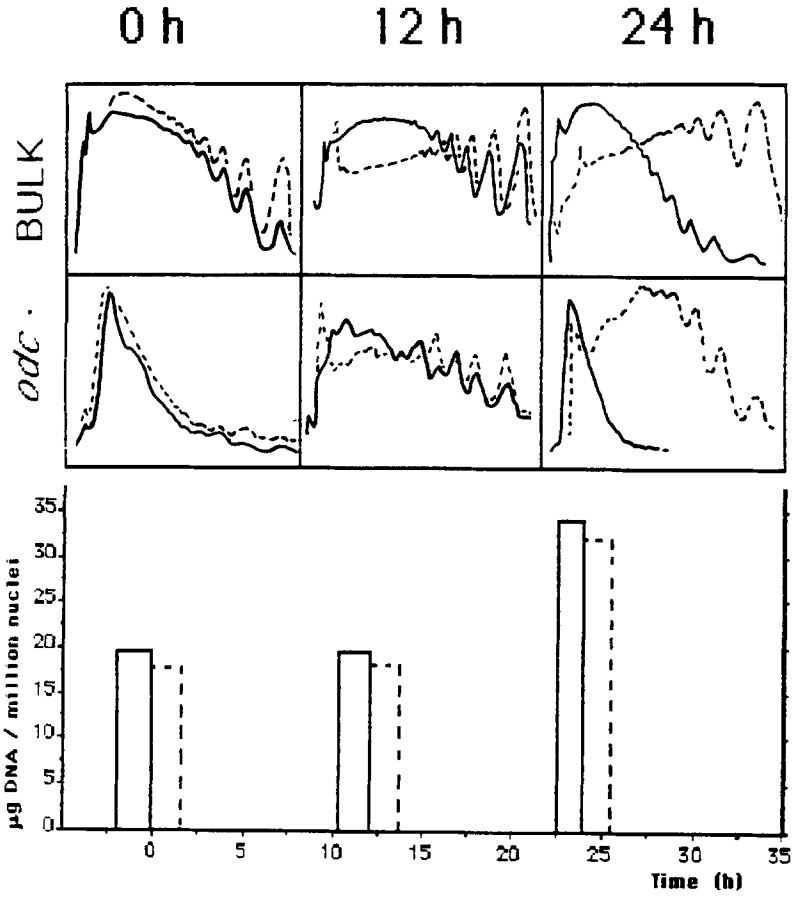

Figure 5. Densitometric scans of nucleosomal organization of the bulk chromatin and *odc* gene from synchronized N1 and E4 cells. The scans representing the 5-min digestion patterns shown in Fig. 4 were generated from photographs (negatives for bulk chromatin) or autoradiograms of the Southern blots hybridized with nick translated probes specific to the *odc* gene. The solid and dashed lines indicate the nucleosomal organization of N1 and E4 cells, respectively. The relative DNA/nucleus ratio of N1 and E4 nuclei are shown as bars with solid or dashed contours, respectively. The x-axis indicates time of serum stimulation in hours.

during the cell cycle. The first peak occurred in mid G_1, the second peak before the maximal DNA synthesis, and the third peak in the G_2 phase (data not shown).

Nucleosomal Organization of Chromatin during the Cell Cycle

To study the chromatin structure of the N1 and E4 cells at different phases of the cell cycle, nuclei were isolated from the cells at 0, 12, or 24 h after serum stimulation and subjected to MNase digestion.

In N1 cells chromatin of the G_1 (12 h) nuclei was markedly more sensitive to MNase digestion than chromatin of the G_0 nuclei (Fig. 4). As the cells traversed from the G_1 to the S phase their chromatin underwent a condensation as judged from the highly MNase-resistant nucleosomal organization in the S phase (24 h) (Fig. 4). Chromatin of the E4 cells displayed somewhat different changes. The most MNase-resistant nucleosomal organization in E4 cells was found after serum starvation, with an increase in sensitivity to MNase after readdition of serum (Fig. 4). Unlike in N1 nuclei, the chromatin of E4 nuclei in mid-S phase (24 h) was as sensitive to the MNase digestion as chromatin from the G_1 nuclei (Fig. 4). Thereafter, in late S and G_2 phase a gradual condensation of the E4 cell chromatin was observed (data not shown).

Fig. 5 illustrates densitometric scanning results of the gel run of the MNase digests in Fig. 4. The digestion patterns show the dynamic behavior of chromatin during the course of serum stimulation. It is notable that, irrespective of the stage of the cell cycle, chromatin from the E4 nucleus (*dashed lines*) is digested significantly faster by MNase than that of the N1 nucleus (*solid lines*). The greatest difference in chromatin digestion between the N1 and E4 cells is seen in S phase (24 h after serum stimulation) when the transformed cells show persisting chromatin decondensation in contrast to the normal cells. A clear difference in the digestion rates between the cell lines is also seen in the G_1 phase.

Nucleosomal Organization of the odc Gene during the Cell Cycle

We, and others, have earlier shown that the transcription of the *odc* gene is increased after serum stimulation in the G_1 phase of the cell cycle (Hölttä et al., 1988; Katz and Kahana, 1987). We therefore studied whether the transcriptionally active *odc* gene carries a nucleosomal organization at the stage when the nucleosomal organization of bulk chromatin is most decondensed. Southern hybridization of the MNase digests after electrophoretic separation, blotting, and hybridization with a nick-translated pODC16-plasmid showed that chromatin of the *odc* gene in both N1 and E4 cells carries a nucleosomal organization in all phases of the cell cycle. However, in the G_1 phase the chromatin of the *odc* gene is much more sensitive to the MNase digestion than in the G_0 phase (Fig. 5). In the S-phase, the chromatin of ODC in N1 cells shows an extensive condensation, unlike in the E4 cells (Fig. 5). Thus, chromatin of the coding and/or 3′ regions of the *odc* gene behaves in a manner similar to the bulk chromatin.

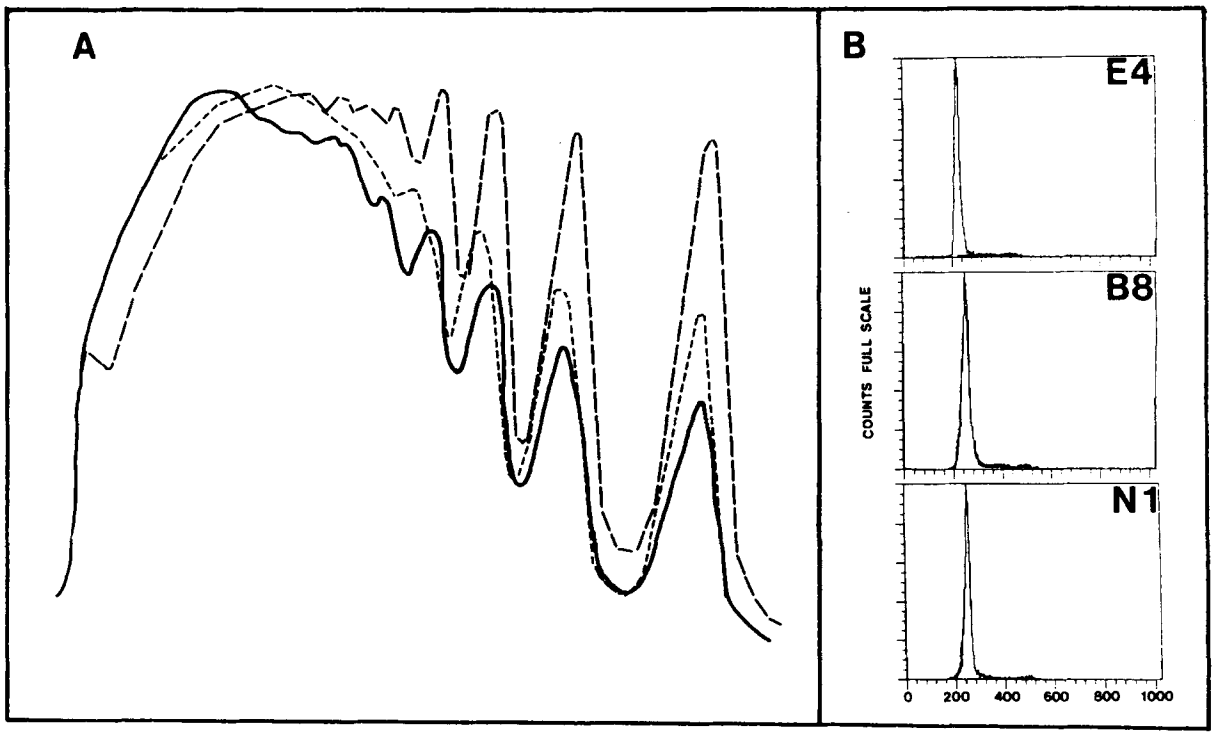

Figure 6. Densitometric scan of nucleosomal organization of the bulk chromatin from normal c-Ha-*ras* protooncogene-transfected (*B8*) cells in comparison to that of N1 and E4 cells. After serum starvation for 24 h, nuclei were isolated from the normal N1 cells, mutant *ras* expressing E4 cells and normal *ras* expressing B8 cells, digested with MNase and analyzed as in Figs. 4 and 5. The solid and dashed lines indicate the nucleosomal organization of N1 and E4 cells, respectively, and the dotted line indicates the nucleosomal organization of B8 cells (*A*). *B* shows the distribution of cellular DNA contents as analyzed by flow cytometry.

The Nucleosomal Organization of Bulk Chromatin from Normal c-Ha-ras Protooncogene-transfected Cells

To see whether the chromatin decondensing effect is a specific property of the mutant c-Ha-*ras* $^{Val\ 12}$ oncogene, we also performed comparative chromatin analyses with cells transfected with the normal c-Ha-*ras* protooncogene (designated B8) (Hölttä et al., 1988). For this study, N1, E4, and B8 cells were synchronized by serum starvation (see Fig. 6 *B*) and stimulated to reenter the cycle as described above. A synchronous progress through the cell cycle was confirmed by ^{3}H-methylthymidine incorporation measurements and flow cytometry (data not shown). We found that in all phases of the cell cycle the chromatin from the normal c-*ras* expressing B8 cells displayed an intermediate accessibility to MNase as compared to the chromatin from the mutant c-*ras* expressing E4 cells and normal N1 cells. This is illustrated by the densitometric scans of a gel run of the MNase digested G_0 nuclei (Fig. 6 *A*).

Discussion

Although it is generally assumed that chromatin first decondenses in the beginning of the cell cycle, and then condenses as the cell approaches mitosis, the behavior of chromatin at the DNA level has not been elucidated until recently by using heparin (Hildebrand and Tobey, 1975), actinomycin D (Pederson, 1972), micrococcal nuclease (Caplan et al., 1978), deoxyribonuclease I (Pederson, 1972; Caplan et al., 1978; Prentice et al., 1985), or EM (Lepault et al., 1980). In this work we show by using micrococcal nuclease as a probe that chromatin of the normal cells displays continuous structural modifications during the cell cycle. Densitometric scanning of the photographs of digestion patterns allowed a precise estimation of the degree of chromatin digestion. Our results with MNase-digested nuclei of NIH-3T3 fibroblasts synchronized by serum starvation are in general agreement with those of Prentice et al. (1985) who analyzed the chromatin structure of CHO cells synchronized by isoleucine deprivation and hydroxyurea by measuring the release of acid-soluble nucleotides from DNase I-digested nuclei. Similarly to our MNase-sensitivity assays, they found that chromatin showed the highest DNase I sensitivity in the G_1 phase, after which it started to condense during the S-phase.

There are only a few comparative studies on the organization of chromatin between normal and transformed cells. Moreover, the cells studied have not been strictly related to each other (Compton et al., 1976; Hartwig, 1982; Linskens et al., 1987). The putative DNA loop size in a leukemic lymphoblast (Hartwig, 1982) and transformed BHK cell (Linskens et al., 1987) has been reported to differ from that of a nontransformed cell. However, it should be noted that the differences may also be due to different growth rates of the normal and transformed cells as recently reported by Chen and Srivastava (1986). They found that DNase I and MNase digest more rapidly chromatin from exponentially growing leukemic cells than chromatin from unstimulated, resting lymphocytes, but also that phytohemagglutinin stimulation of the lymphocytes resulted in disappearance of differential digestion kinetics.

In this work we show that the nucleosomal organization of the c-Ha-*ras* oncogene-transformed fibroblasts (E4) is more

decondensed than that of the parental normal fibroblasts (N1). In addition, we found that chromatin from normal c-*ras* protooncogene-transfected cells (B8) displays an intermediate sensitivity to MNase in relation to the chromatin from the normal N1 cells and mutant *ras*-transformed E4 cells. Whether a very high expression of the c-*ras* protooncogene capable of transforming the cells would cause a more marked decondensation of the chromatin remains an interesting object of further study. The differences observed in the nucleosomal organization of chromatin were not due to the cells being in different phases of the cell cycle (Prentice et al., 1985) as shown by synchronization experiments. In all cell cycle phases, the nucleosomal organization of the E4 cells appeared to be significantly more decondensed than that of the N1 and B8 cells. The greatest differences in the level of chromatin condensation between the E4 and N1 cells are seen in the S phase. In normal cells the decondensation of chromatin in G_1 is followed by a marked condensation in the S phase, but in *ras*-transformed cells the decondensation state stays. The same result was also obtained when using aphidicolin, an inhibitor of eukaryotic DNA replication, for the synchronization of *ras*-transformed and normal cells (Laitinen, J., and E. Hölttä, unpublished data).

Many nuclease studies are obscured due to the presence of endonucleolytic nucleases (Vanderbildt et al., 1982; Shah and Lea, 1987). Autodigestion is a major problem for example when the chromatin structure of liver cells is studied by using exogenic nucleases as probes (Vanderbildt et al., 1982). However, in our study, autodigestion was not detected and thus did not interfere with the results.

Southern blot analyses using gene-specific probes showed that DNA of *odc*, c-*myc*, and *gapdh* genes (at their coding and/or 3′ regions) have a nucleosomal organization. The chromatin of the *odc* gene appeared to have a nucleosomal organization in all phases of the cell cycle both in N1 and E4 cells. Similar results have been reported for the 3′ coding region of the *histone H4* gene (Moreno et al., 1986). In contrast, the 5′ region of the H4 gene displays a disrupted nucleosomal organization in the S phase as the gene is transcribed (Moreno et al., 1986). As our results show that the nucleosomal organization of the coding and 3′ end regions of the c-*myc* and *odc* genes appear to accompany that of the bulk chromatin, it is to be expected that the expression of these genes must be regulated at their 5′ upstream regions. In addition, rapid and reversible changes in nucleosome structure of the protooncogenes c-*fos* and c-*myc* have been shown to accompany their transcriptional activation in BALB/c 3T3 cells (Chen and Allfrey, 1987). Studies on the nucleosomal organization of the 5′ region of these genes, which are the sites of main transcriptional regulation will thus be of great interest.

Altogether, to our knowledge this is the first report showing that cell transformation is associated with a decondensation of nucleosomal organization. Recent studies from our and other laboratories (Wasylyk et al., 1988) have suggested that c-Ha-*ras* $^{Val\ 12}$ oncogene activates the expression of several growth-related genes, and this might occur through binding of the transacting factors (like AP-1 transcription factor) to the relevant gene enhancers. The nature of the factor(s) which renders chromatin of the c-Ha-*ras* $^{Val\ 12}$ oncogene-transformed cells (E4) more MNase sensitive than that of the normal cells (N1) remains yet unresolved. Therefore, studies on, e.g., the amounts of the histone H1 variants (Biard-Roche et al., 1982) that are responsible for the higher order organization of chromatin (Igo-Kemenes et al., 1982; Weintraub, 1984; Widom and Klug, 1985; Falsenfeld and McGhee, 1986; Allan et al., 1980; Cole et al., 1977) are of particular interest. Recently, it was shown that the histone H1 binds to the putative recognition site of the transcription factor NF-1 in the mouse α_2(I) collagen promoter (Ristiniemi and Oikarinen, 1989). Thus, it is tempting to speculate that removal of histone H1 and consequent decondensation of chromatin is of primary importance for enhancement of expression of transformation-sensitive genes by specific transcription factors.

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