Vol. 128, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1985 Pages 226-232

THE ASSOCIATION OF HUMAN c-Ha-ras SEQUENCES WITH CHROMATIN AND NUCLEAR PROTEINS

Usha N. Kasid, Chris Hough, Peter Thraves,*
Anatoly Dritschilo, * and Mark Smulson

Department of Biochemistry and the *Division of Radiation Oncology
Vincent T. Lombardi Cancer Research Center
Georgetown University Medical Center
Washington, D.C. 20007

Received February 5, 1985

As a step towards the understanding of possible relationship between chromatin organization and regulation of the oncogene expression, we have investigated the chromatin structure of one of the more frequently activated oncogenes, c-Ha-ras, in HeLa-S3 cells. This was accomplished by isolation of the chromatin fractions (soluble and insoluble) after micrococcal nuclease digestion of purified nuclei and probing for the distribution of ras sequences. The polynucleosomal fraction was further resolved by sucrose gradient sedimentation. Southern-blot hybridization of the DNA isolated from various fractions yielded following results: (1) c-Ha-ras seqences segregated predominantly in the lysate fraction. (2) Unlike the *B*-globin (transcriptionally inactive) sequences, ras-H associated chromatin lacked typical nucleosomal packaging. Furthermore, since post-translational modifications of nuclear proteins have been suggested to modulate the nucleosome structure during DNA transcription and replication, ras sequences, in polynucleosomes immunofractionated on anti-poly (ADP-Ribose) Sepharose were also examined. The data suggested that the major class of this oncogene sequence exists in chromatin more distal to the sites of this particular chromatin modification.

Much of the intense research of the past few years has been concentrated on the issue of oncogene activation. Whether the transforming potential of the gene is activated by qualitative changes in gene structure or by quantitative changes in expression is not yet clearly known. Differential sensitivity to nucleases has occasionally been shown to distinguish the chromatin structure of the actively transcribing genes from those that are inactive. However, to our knowledge, limited information is available on the nature of nuclear proteins associated with oncogene sequences, or the chromatin configurations of these genes. Thus, we felt a study of the chromatin structure of an oncogene and its comparison with that of an inactive gene should provide an insight into this topic. In the present study we have pursued this objective with respect to the c-Ha-ras oncogene in HeLa-S3 cells.

MATERIALS AND METHODS

Preparation of HeLa Chromatin: HeLa-S3 cells were maintained at 37°C in spinner flasks in Eagle's medium containing 10% fetal calf serum. The nuclei were isolated by the method of

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Sporn et al. (1) and washed with 0.25 M sucrose/5 mM Tris HCl, pH 7.5/1mM $CaCl_2$/80 mM NaCl containing 0.3% Triton X-100. The purified nuclei were treated with micrococcal nuclease (30 U/10^8 nuclei) for 3 min at 37°C by the method of Butt and Smulson (2). 10-20 A_{260} units (0.5 ml) of isolated chromatin were layered on to 10-30% linear sucrose gradient containing 1mM sodium phosphate (pH 6.8), 0.2 mM EDTA, and 80 mM NaCl. The gradients were centrifuged in a Beckman SW40 rotor at 40,000 rpm for 4.5 h at 4°C.

Hybridization: DNA fragments were isolated as described (3), electrophoretically resolved on 0.7% agarose gel, stained with ethidium bromide and blot transferred to nitrocellulose (Schliecher and Schuell, BA 85) by the method of Southern (4). The nitrocellulose filters were seriallly hybridized to ^{32}P-labelled c-Ha-ras and *B*-globin gene probes as described (5,6), and autoradiographs were developed using Kodak XAR-5 films.

Immunofractionation: Oligonucleosomes (approximately 1.5 A_{260}) were labelled with [^{32}P] NAD and immunofractionated on anti-poly (ADP-ribose)IgG-Sepharose column as described earlier by us (7). The unbound fractions were collected and pooled after washing the column with phosphate buffered saline. The bound material (modified nucleosomes) was eluted with 1.6 M KSCN, dialyzed and pooled.

RESULTS

The Ras Gene, Nuclease Digestion, and Chromatin Fractionation

HeLa cell nuclei ($4x10^8$) were digested with micrococcal nuclease (30 U/10^8 nuclei) for 3-5 minutes. This represented a mild digestion with the nuclease, and corresponded to approximately 7% acid soluble digestion. The digested nuclei were centrifuged at 5,000 rpm and the supernatant (S1) represented cleaved, small chain length oligonucleosomes, highly susceptible to micrococcal nuclease and, as recently described by Lawson and Cole (8), depleted of histone H1 and enriched in high mobility group proteins. The pelleted nuclei were lysed, centrifuged (7000 rpm) and the supernantant (S2), representing nuclease sensitive regions of chromatin and containing polynucleosomes of various chain lengths, was collected. The remaining pellet from the centrifugation (P) represents the regions of chromatin inaccessible to micrococcal nuclease early in digestion, polynucleosomes that sediment at 7,000 rpm, and probably nuclear matrix associated forms of chromatin (9).

DNA was extracted from these three fractions from two separate experiments and equal A_{260} units were electrophoresed on 0.7% agarose gels and subsequently stained with ethidium bromide. The S1 fraction, as indicated above, was composed mainly of small chain lengths of nucleosomes whereas the other two fractions (S2 and P) contained 200 bp arrays of nucleosomal chain lengths consisting of mononucleosomes up to 7-8 resolveable and possibly even higher chromatin units.

The electrophoresed DNA was transferred to nitrocellulose and first hybridized to a [^{32}P] labelled human EJ Bladder carcinoma oncogene probe (c-Ha-ras) (Fig.1). After autoradiography the nitrocellulose sheet examined in Fig. 1 was washed free of radioactivity and subsequently

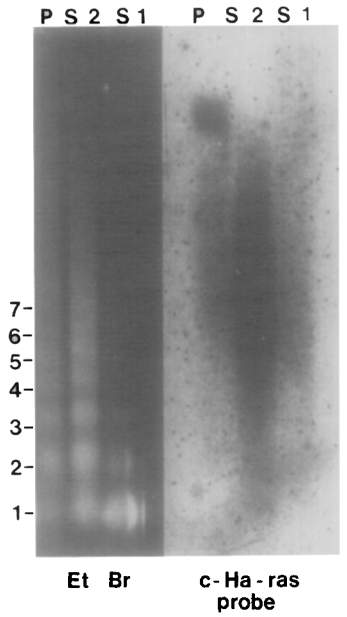

Fig.1 Predominant segregation of c-Ha-ras in the lysate fraction of chromatin sequence. HeLa DNA was isolated from various chromatin fractions (S_1, S_2 and P) eletrophoresed (10 ug/lane) on 0.7% agarose gel, stained with ethidium bromide and hybridized to a [^{32}P]-labelled c-Ha-ras probe, 6.6 Kb Bam HI fragment of EJ ras^H, using 5 X SSC, 50% formamide at 42°C. The blot was washed to a final stringency of 0.3 X SSC at 63°C, dried and exposed to XAR-5 film at -80°C for 3 days. [Numbers 1-7 are sizes of oligonucleosomes].

hybridized to a second probe. We chose a 4.4 kb insert containing the entire human *B*-globin gene sequence (10). *B*-globin is not transcribed in HeLa cells (A. Nienhuis, personal communication).

Significant differences were noted in DNA-protein interaction as determined by the hybridization of the same nitrocellulose sheet with the c-Ha-ras gene. Ras gene domains in chromatin appeared to be highly sensitive to nuclease digestion since only negligible hybridization of probe to this gene was noted in the nucleosomal particles, isolated in the S1 fraction (Fig. 1). Secondly, while the inactive *B*-globin gene hybridized directly with the nucleosomal 200 bp repeat bands (See Fig. 2), the c-ras gene hybridization with the DNA in both the S2 and pellet fractions appeared as a smear. The chromatin organization of the ras gene therefore appeared to display characteristics recently reported to correspond to transcriptionally active gene sequences (11). Thirdly, high proportions of the regions of chromatin proximal to the ras sequences were moderately nuclease sensitive as evidenced by their fractionation into the S2 fraction rather than the pellet, a nuclease digestion property of active genes. Since the c-ras chromatin appeared to possess a non-nucleosomal conformation, the S2 fraction was further

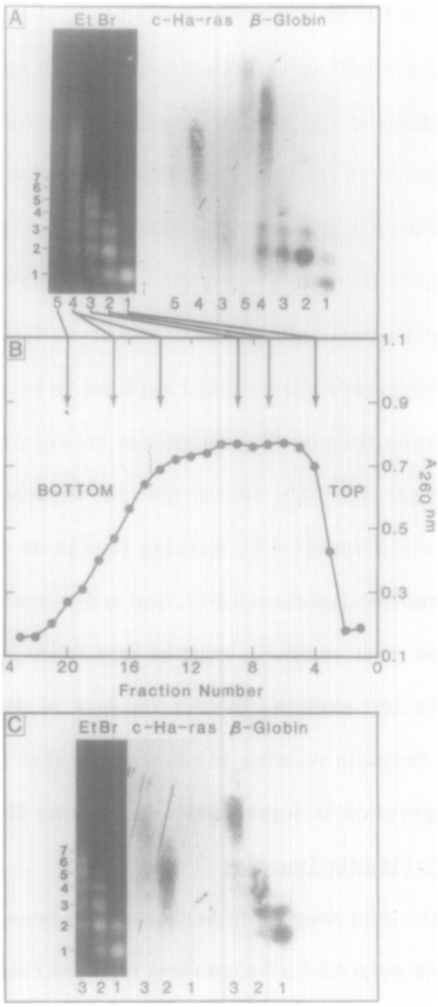

Fig.2 c-Ha-ras gene sequences lack typical nucleosomal packaging. 0.5 ml of isolated chromatin (S_2, 10 A_{260})in two separate experiments (A and C) was applied onto two 10-30% linear sucrose gradients and oligonucleosomes of various sizes were isolated as explained in Materials and Methods. Panel B, Sucrose gradient profile of S_2 from the first experiment (A). The DNA extracted from purified oligonucleosomes was size fractionated on 0.7% agarose gel, stained with ethidium bromide, blot transferred onto nitrocellulose and first hybridized to [^{32}P]-labelled c-Ha-ras probe under conditions explained in legend to Fig. 1. The same filter was (after denaturation of the first probe) hybridized to [^{32}P]-labelled *B*-globin gene probe (4.4 Kb pstI, note that the nucleosomal repeats appear on autoradioagraph after hybridization with B-globin probe in both experiments A and C). The enlargement of ethidium bromide stained pictures differs from that of their autoradiographs. [Numbers 1-7 represent the sizes of nucleosomes].

purified by sucrose density gradients to resolve more clearly whether this gene was associated with nuclear proteins yielding particles of differing sedimentation values.

Analysis of c-ras Sequences in Purified Oligonucleosomes

The polynucleosomes obtained in the S2 fraction were sedimented in a 10-30% sucrose gradient for 4.5 hours at 40 K rpm. Particles from 5-80S were resolved on the sucrose gradient

(Fig. 2B). The DNA was extracted from 5 regions of the gradient representing mononucleosomes to oligonucleosomes comprised of 15-20 units (approximately 3730 bp). Two independent analyses were performed (Fig. 2A and C). The DNA was electrophoresed on agarose gels, stained with ethidium-bromide, transferred to nitrocellulose and hybridized first to the ^{32}P-labelled c-Ha-ras probe. As before, after autoradiography, the sheet was washed free of the hybridized first probe, and subsequently hybridized to the B-globin probe. Similar hybridization patterns of c-Ha-ras to those shown in Fig. 1 were confirmed with these purified oligonucleosomes. *B*-globin gene sequence appeared to be organized with nuclear proteins to yield a relatively typical nucleosomal 200 bp ladder as evidenced by the similarities between the hybridization and the ethidium bromide stain of this gel (Fig. 2C). In contrast, ras sequences, in resolved chromatin subfractions, hybridized with ethidium bromide staining regions as well as the interband areas of the gel. DNA isolated from gradient fractions 14-17, had sedimentation values of approximately 40s on the sucrose gradient, and thus could not exist as free DNA in the S2, but rather associated with some type of nuclear protein. In fact, the lack of distinct hybridization, corresponding to the ethidium bromide staining bands suggests that this oncogene is not packaged in a typical nucleosomal configuration in logarithmically growing HeLa cells.

Immunofractionation of Ras Chromatin Domains

An antibody to poly(ADP-Rib) coupled to Sepharose has been useful to isolate selectively polynucleosomes undergoing the poly(ADP-ribosylation) reaction from the bulk chromatin (7).

The polynucleosomes purified by sucrose gradient centrifugation (Fig. 2) were incubated in vitro with 10 uM[^{32}P] NAD and immunofractionated on the anti-poly (ADP-Rib) column. Approximately 50% of the [^{32}P]-poly (ADP-ribosylated) radioactivity was associated with the

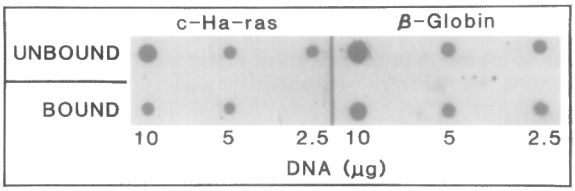

Fig.3 Partial retention of c-Ha-ras sequences in modified nucleosomal domains. Oligonucleosomes (8-10 units) were poly (ADP-ribosylated) with [^{32}P] NAD. The labelled nucleosomal sample was passed through a small column of Sephadex G-25 to remove unincorporated NAD and immunofractionated on anti-poly (ADP-ribose) IgG-Sepharose 4B. The DNA was extracted from unbound and bound fractions, serially diluted so as to dot blot indicated amounts onto nitrocellulose. The filter was serially hybridized first with [^{32}P]-labelled c-Ha-ras probe followed by hybridization with [^{32}P]-labelled B-globin probe.

bound fractions of chromatin was achieved by this procedure. DNA from unbound and bound fractions were dot-blotted to nitrocellulose using serial dilutions (Fig. 3). The data suggests that the ras sequences may exist in chromatin more distal from poly (ADP-ribosylated) regions, since quantitatively more (3 fold) hybridization of the ras probe per ug DNA was found in the unbound fractions.

DISCUSSION

A number of reports have recently appeared which attribute the increased transcriptional activity of cellular oncogenes to local alterations of chromatin structure associated with the insertion of the LTR or other proviral sequences (12). The similarity of hybridization pattern of c-ras sequences to that of "normal" actively transcribing eukaryotic genes (Figs. 1 and 2) suggests that the organization of a cellular oncogenic sequence as detected by micrococcal nuclease bears a relationship to the "normal" functional organization of eukaryotic DNA by nucleosomse. This hypothesis is further strengthened by the close conservation of the cellular oncogenes probably due to the essential role their products play in cell differentiation and regulation of cell division. Secondly in the present study we have analyzed the association of c-Ha-ras sequences with poly ADP-ribosylated domains of chromatin. The condensation/decondensation of chromatin organization by poly (ADP-ribosylation) could be one of the mechanisms by which nuclear events such as DNA replication, repair and transcription are favoured (2). Present observations suggest that a majority of human Ha-oncogene sequences are located distal to the sites of modified nuclear proteins.

ACKNOWLEDGEMENTS

We are deeply indebted to Drs. R. Weinberg (MIT) and A. Neinhaus (NIH) for probes. We also thank Sharon Stewart for the excellent typing of this manuscript. This research was supported by National Institutes of Health grants CA13195 and CA25344, Bristol Myers Company Research Development in Cancer Research Funds, and Radiation Medicine Departmental support.

REFERENCES

1. Sporn, M.B., Berkowitz, D.M., Glinski, R.P., Ash, A.B., and Stevens, C.L. (1969). Science 164, 1408-1410.
2. Butt, T.R., and Smulson, M. (1980). Biochemistry 19, 5235-5242.
3. Mullins, D.W., Giri, C.P., and Smulson, M.E. (1977). Biochemistry 16, 506-513.
4. Southern, E.M., (1975). J. Mol. Biol. 98, 503-517.

5. Kasid, U.N., Stefanik, D., Lubet, R., Dritschilo, A., and Smulson, M. (1985). Submitted for publication in Proc. Natl. Acad. Sci. USA.
6. Hough, C.J., and Smulson, M.E. (1984). Biochemistry, 23, 5016-5023.
7. Malik, N., Miwa, M., Sugimura, T., Thraves, P.J., and Smulson, M.E. (1983). Proc. Natl. Acad. Sci. USA 80, 2554-2558.
8. Lawson, G.M., and Cole, R.D. (1982). J. Biol Chem. 257, 6576-6580.
9. Goldberg, G.I., Collier, I., and Cassel, A. (1983). Proc. Natl. Acad. Sci. U.S.A. 80, 6887-6891.
10. Lawn, R.M., Efstratiadis, A., O'Connell, C., and Maniatis, T. (1980). Cell 21, 647-651.
11. Smith, R.D., Seale, R.L., and Yu, J. (1983). Proc. Natl. Acad. Sci. U.S.A. 80, 5505-5509.
12. Schubach, W., and Groudine, M. (1984). Nature 307, 702-708.