DNA SYNTHESIS BY AN INSOLUBLE CHROMATIN FRACTION ASSOCIATED WITH THE NUCLEAR MEMBRANE OF ANIMAL CELLS

Masako Yoshikawa-Fukada[1] and J. D. Ebert

Department of Embryology, Carnegie Institution of Washington
115 West University Parkway, Baltimore, Maryland 21210

Received February 18, 1971

Summary - An insoluble chromatin fraction prepared from Chinese hamster, HeLa, and baby hamster kidney (BHK) cells is capable of DNA synthesis in the absence of added primer. The DNA in the insoluble chromatin hybridizes with the RNA of Rous sarcoma virus to a substantially greater degree than does the DNA of the soluble chromatin fraction. Studies of the DNA produced *in vitro* suggest that the insoluble fraction may be a DNA-synthesizing rather than a terminal-repairing system.

Introduction - The RNA of Rous sarcoma virus (RSV-RNA) hybridizes with DNA's from eukaryotes and from oncogenic viruses (1-4). Both 70S and 4S RSV-RNA's contain nucleotide sequences homologous to those in cellular DNA, and which have been shown, by competition techniques, to be distinct from sequences in cellular RNA's, whether ribosomal or soluble (5). RSV-RNA hybridized to all of the DNA's analyzed has a closely similar base composition; moreover there are close similarities in melting temperatures (1-3,5).

In exploring the role of the "oncogenic viral sequences," we have centered our attention on the chromatin associated with the nuclear membrane. A common property of all of the tumorigenic viruses is their ability to induce and stimulate cellular DNA synthesis (4). The kinetics of formation of hybrids between cellular DNA and RSV-RNA, under stringent conditions, suggests that the homologous DNA belongs in the "repeated DNA sequences," further suggesting that it may have a regulatory role (5,6). These oncogenic sequences appear to be dispersed throughout the karyotype (3,4). Some evidence suggests that in eukaryotes, DNA synthesis is initiated in association with the nuclear membrane (7,8), just as it is associated with the cellular membrane in bacteriophage and bacteria (9-11).

---

1. Present address: Institute for Virus Research, Kyoto University, Japan

Materials and Methods - Preparation of insoluble chromatin associated with the nuclear membrane (SP) - Chinese hamster cells (provided by M. Elkind) and HeLa cells were grown in suspension in MEM 510 containing 5 percent tryptose phosphate broth and 10 percent calf serum (Flow Laboratories). The initial inoculum was 2 x 10^5 cells/ml. After two to three days' incubation, when the cells were growing exponentially (6-7 x 10^5 cells/ml), they were synchronized by treatment with excess (2.5 mM) thymidine for either 12 or 18 hours, for Chinese hamster and HeLa cells, respectively, after which the cells were collected, washed with Hanks' salt solution and cultured at 1-2 x 10^6 cells/ml in fresh medium for a further six hours. This period corresponds to early and middle S phase of synchronized cells as judged by pulse labeling with ^{3}H-thymidine.

BHK 21 cells (line C 13-404, provided by H. V. Aposhian) were grown in the same medium but in monolayer cultures (5-8 x 10^6 cells/15 cm Falcon plastic Petri dish). When cultures reached confluency, cells were transferred to large rolling bottles. After 48 to 72 hours' incubation, the medium was replaced with fresh medium (the same). The cells were collected after 12 to 18 additional hours by trypsinization (0.25 percent trypsin in Hanks' solution without Ca^{++}).

The following steps were performed on all cells at 0^o to 4^o C., except as noted. (1) Cells were washed once with KMN solution (5 mM KCl, 1.5 mM $MgCl_2$, 140 mM NaCl), and suspended for 10 minutes in TM buffer (10 mM Tris HCl, pH 7.6, 6 mM 2-mercaptoethanol, 2 mM $MgCl_2$) at a final concentration of 10^7 cells/ml. Under these conditions the cells swell rapidly. (2) Triton X 100 was added to a final concentration of 1.0 percent and the swollen cells were shaken vigorously by hand for two to three minutes, after which they were disrupted by passage through a 22 gauge needle (3-5 strokes), with 80 percent of the nuclei being freed. (3) Nuclei and the remaining intact cells were sedimented by centrifugation at 1000 rpm for five minutes. The resulting fractions were operationally designated "nuclear" and "cytoplasmic." (4) The "nuclear" fraction was suspended in TM buffer containing 0.25 M sucrose (nuclei from 1-2 x 10^9 cells/20 ml of solution) and purified by centrifuging through 10 ml of 2 M sucrose in TM buffer

at 24,000 rpm for 45 minutes, using a Spinco preparative ultracentrifuge with the SW 25.1 rotor. (5) The sedimented nuclei were washed once with MM solution (2 mM 2-mercaptoethanol, 0.125 mM $MgCl_2$) containing 0.25 M sucrose, and suspended in MM solution at a concentration of 5-10 x 10^8 nuclei/10 ml. (6) This suspension was then sonicated in a Bronson sonicator (power 4, 5-6 mA) for 30 seconds and (7) centrifuged at 10,000 x g for 30 minutes. The resultant pellet was suspended in MM solution and used as the insoluble chromatin or SP (sonic precipitate) fraction. The supernatant, or soluble chromatin, is designated the SS (sonic supernatant) fraction.

The "cytoplasmic" fraction (step 3) was centrifuged again at 12,000 rpm for 30 minutes, the resulting supernatant fluid being fractionated on Sephadex G 100 (12). The capacity of each fraction for DNA synthesis in the presence of DNA was examined, with heat-denatured calf thymus DNA (Worthington Biochemicals Corporation) as primer. The most active fractions were pooled and used as "cytoplasmic DNA polymerase."

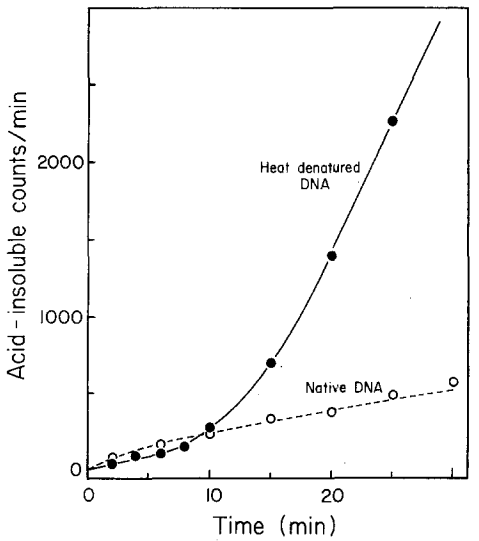

Fig. 1 Time course of ^3H-TTP incorporation by cytoplasmic DNA polymerase and exogenous primer DNA. Reaction mixture is the same as in Table 1, except that one set (broken line) contained 30 μg native, rather than denatured, DNA. Each point is the average of two determinations. The lag cannot be observed if the mixture is held at 4° C. after the addition of the enzyme. By reaction time we mean precisely the period from the addition of enzyme to the addition of trichloracetic acid.

Results - Characteristics of the SP fraction - This fraction is capable of DNA synthesis -- the incorporation of thymidine triphosphate (^{3}H-TTP) into the acid-insoluble fraction -- in the absence of added primer DNA. In Table 1 we compare the properties of the SP fraction with those of the cytoplasmic DNA polymerase which requires added primer DNA for the incorporation of ^{3}H-TTP into the acid insoluble fraction.

When ^{3}H-TTP is incorporated by the cytoplasmic DNA polymerase fraction, there is an initial lag of five to eight minutes (Fig. 1). This lag is not observed when the cytoplasmic DNA polymerase fraction is preincubated with heat-denatured DNA for 10 minutes (Fig. 2), suggesting that the cytoplasmic DNA polymerase must form a complex with the primer to initiate DNA synthesis. No

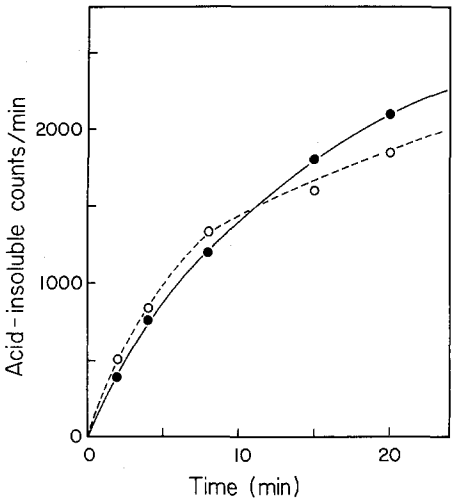

Fig. 2 Time course of ^{3}H-TTP incorporation by preincubated cytoplasmic DNA polymerase and exogenous DNA. Cytoplasmic DNA polymerase (100 μg protein) was incubated in TM buffer with heat-denatured calf thymus DNA for 10 minutes at 4° C. (open circles) or at 37° C. (closed circles), after which substrate and ^{3}H-TTP was added as in Table 1 and the experiment was performed as in Fig. 1. Each point is the average of two determinations.

lag is observed in ^{3}H-TTP incorporation by the SP fraction, incorporation reaching a plateau in 10 to 15 minutes (Fig. 3). If at that point, primer is added, DNA synthesis is reinitiated. In contrast, the addition of cytoplasmic DNA polymerase to the SP fraction is not stimulatory.

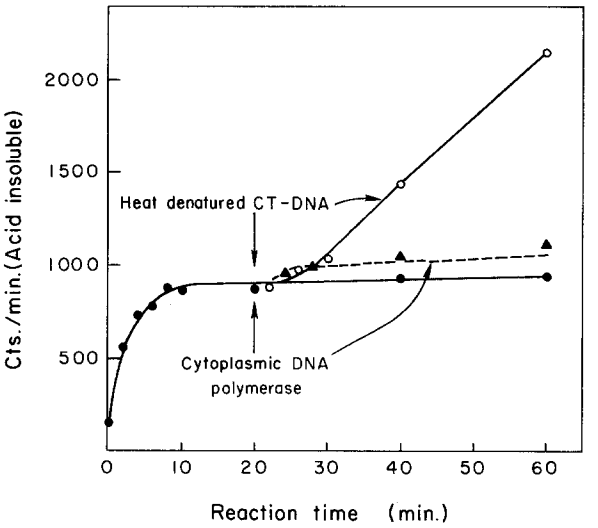

Fig. 3 Time course of ^{3}H-TTP incorporation by SP fraction. See Table 1 for reaction mixture. After incubation for 20 minutes at 37° C., 30 μg of heat denatured calf thymus DNA was added to one set of tubes, cytoplasmic DNA polymerase (50 μg protein) to another, and TM buffer to a third set. All three sets were then incubated for another 40 minutes at 37° C. Each point is the average of two determinations.

There is a second major difference between the SP and "cytoplasmic DNA polymerase" fractions. Using techniques similar to those described by Jackson et al. (13) we have already shown that DNA extracted from insoluble calf thymus chromatin hybridizes with RSV-RNA to a greater degree than does DNA from soluble chromatin (2). As seen in Table 2, DNA extracted from the SP fraction derived from S phase Chinese hamster cells hybridizes with RSV-RNA from 1.2 to 2.5 times higher than DNA derived from the SS fraction.

Some properties of the product DNA - A fraction of DNA synthesized by the SP fraction in the presence of 5-bromo deoxyuridine triphosphate (BUTP) bands at a greater density in cesium chloride centrifugation than does control DNA or marker (unlabeled DNA of the SP fraction). The DNA synthesized by the SP fraction with ^{3}H-TTP appeared in the same position as marker DNA (Fig. 4A). However when BUTP replaced TTP in the reaction, two peaks were observed, one corresponding to the marker peak and a heavier peak (Fig. 4B). The radioactivity

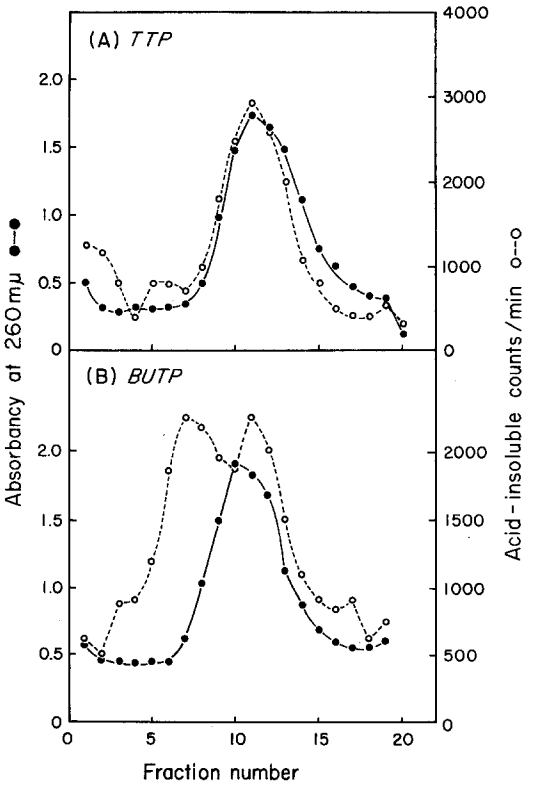

Fig. 4 CsCl centrifugation of DNA's synthesized by SP fraction in the presence of TTP or BUTP. The reaction mixture described in Table 1 was employed, scaled up to 1.25 ml. The SP fraction was prepared from BHK cells (see Materials and Methods). (A) contained ^{3}H-TTP, dATP, dCTP and dGTP. (B) contained BUTP, ^{3}H-dATP (Schwarz BioResearch, 250 μc/11.1 μg), dCTP and cGTP. BUTP was provided by Dr. M. Bessman. The reaction was stopped after 30 minutes by adding EDTA and SDS to final concentrations of 0.02 M and 1 percent respectively. The refractive index was adjusted to 1.4010-1.4015 by adding an appropriate amount of CsCl, the salt concentration was brought to 2 x SSC, and 300 μg of DNA extracted from the BHK-SP fraction was added as a marker. After centrifugation at 30,000 rpm for 68 hours at 20° C. (number 65 rotor), the volume of each fraction was brought up to 0.4 ml with 2 x SSC, the optical density was determined, 3 ml of 5 percent trichloracetic acid was added and the acid-insoluble fraction was collected and counted as previously described.

---

in this experiment was provided by ^{3}H-dATP. These findings suggest that in addition to its dependency on dXTP (Table 1), the SP fraction can incorporate significant amounts of BUTP resulting in newly synthesized DNA that is significantly heavier than the control. Thus the SP fraction appears to be a DNA-synthesizing rather than a DNA-terminal repairing system.

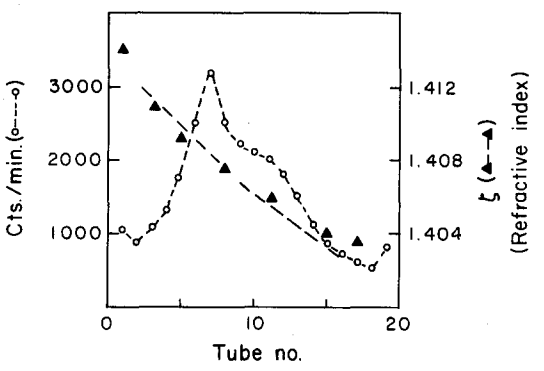

Fig. 5 Alkaline CsCl centrifugation of DNA synthesized by the SP fraction. The reaction mixture was the same as described in Fig. 4(A) except that it was derived from Chinese hamster cells. Incubation proceeded for 30 minutes at 37° C. The reaction was stopped by adding NaOH and EDTA at final concentrations of 0.1 N and 0.01 M, respectively. The refractive index was then adjusted to 1.4065 by adding CsCl in 2 x SSC containing 0.1 N NaOH and 0.01 M EDTA. After centrifugation at 33,000 rpm for 60 hours at 20° C. (no. 65 rotor), the refractive index of each fraction was determined, the solution was neutralized, adjusted to 4 x SSC and trapped on a Millipore filter. After drying, the filters were counted in toluene scintillator.

The DNA synthesized by the SP fraction was also studied by alkaline cesium chloride gradient centrifugation. In alkaline CsCl (Fig. 5), the pattern suggests that the GC content of the two strands may be different, thus presenting the possibility of separating the strands for further characterization of this DNA.

Discussion - An insoluble chromatin fraction (SP) derived from the nuclear membrane of several types of mammalian cells is capable of DNA synthesis in the absence of added DNA primer. In the SP fraction, DNA and DNA polymerase appear to exist as an active complex, for DNA synthesis starts immediately upon the addition of ^{3}H-TTP. If, after 30 minutes' incubation, the SP reaction mixture is centrifuged for 30 minutes at 10,000 x g, DNA-synthesizing activity, requiring primer, is now recovered in the supernatant, suggesting that once the SP-DNA polymerase finishes reading the associated template DNA, it is released as free enzyme. The finding that RSV-RNA hybridizes to a greater extent with the insoluble, than with the soluble, chromatin is consistent with the hypothesis that the viral oncogenic sequences may be involved in the initiation of

Table 1. DNA synthesis *in vitro*

Source of enzyme		^{3}H-TTP incorporated into acid insoluble fraction (cpm)
(1) Cytoplasmic DNA polymerase	complete system	31335
	-DNA	1284
	-dATP,-dCTP,-dGTP	8986
(2) SP fraction	complete	3509
	-dATP,-dCTP,-dGTP	288
	-Mg^{++}	404
	-2 mercaptoethanol	1820
	+Pancreatic DNase (50 μg/ml)	292
	+Pancreatic RNase (50 μg/ml)	3489
	+Sarkosyl (1%)	332

Complete system (0.25 ml) for (1) consists of 20 μmoles Tris buffer, pH 7.8, 2 μmoles $MgCl_2$, 1 μmole 2-mercaptoethanol, dXTP (each) 50 mμmoles, ^{3}H-TTP (2 μc/mμmole) 1 μc, heat denatured calf thymus DNA 30 μg and cytoplasmic DNA polymerase from HeLa cells (100 μg protein). Complete system for (2) is the same as for (1) except calf thymus DNA was omitted, and SP replaced cytoplasmic DNA polymerase. After incubation for 60 min. at 37° C., the reaction was stopped by adding 3 ml of cold 5% trichloracetic acid containing 0.01 M sodium pyrophosphate. Tubes were kept in an ice bath for at least 30 min., after which the acid insoluble fraction was collected on a Whatman GF/C glass fibre filter, washed three times with 5 ml of cold 5% trichloracetic acid, dried and counted in toluene scintillator. Figures are average counts of three measurements.

replication of cellular DNA. Comings and Mattoccia (14) have presented evidence indicating that a piece of repetitious DNA may occur at or near the beginning of each replicon. Smith (15) has found that in confluent mouse kidney cells, in which DNA synthesis was switched on by polyoma virus infection, the satellite DNA replicated before the remainder of the cellular DNA.

Table 2. Comparison of soluble chromatin (SS) and nuclear membrane associated insoluble chromatin (SP) DNA's

Source of DNA	Hybridized 70S RSV-RNA (cpm)		
	preparation (1)	(2)	(3)
S phase Chinese hamster cell			
SS	676	820	1028
SP	1610	1530	1250

Filters containing 60 μg of SS-DNA of 60 μg SP-DNA extracted from 3 different preparations, were hybridized with 1.5 μg of 70S RSV-RNA (1.1×10^5 cpm/μg) in the presence of 0.5 mg of chick embryo sRNA and 0.5 mg of chick embryo rRNA under the conditions described previously. The figures are average counts of 3 filters.

Acknowledgement - The authors wish to acknowledge the cooperation of Dr. Charles W. Orr and the technical assistance of Delores Somerville and Bessie Smith. The preparation of the manuscript was aided by the constructive criticism of Drs. Orr, R. H. Reeder and R. G. Roeder.

References

1. Yoshikawa-Fukada, M., and J. D. Ebert, Proc. Nat. Acad. Sci., U.S., 64, 870 (196
2. _____ Carnegie Inst. Wash. Year Book, 68, 521 (1970).
3. _____ Carnegie Inst. Wash. Year Book, 69, 583 (1971).
4. _____ BioScience (1971, in press).
5. _____ Proc. Nat. Acad. Sci., U.S. (1971, in press).
6. Britten, R. J., and E. H. Davidson, Science, 165, 349 (1969).
7. Comings, D. E., and T. Kakefuda, J. Mol. Biol., 33, 225 (1968).
8. Pawlowski, P. J., and L. Berlowitz, Exptl. Cell Res., 56, 154 (1969).
9. Hallick, L., R. P. Boyce and H. Echols, Nature, 223, 1239 (1969).
10. Knippers, R., and W. Stratling, Nature, 226, 713 (1970).
11. Fuchs, E., and P. Hanawalt, J. Mol. Biol., 52, 301 (1970).
12. Jovin, T. M., P. T. Englund and L. L. Bertsch, J. Biol. Chem., 244, 2996 (1969).
13. Jackson, V., J. Earnhardt and R. Chalkley, Biochem. Biophys. Res. Comm., 33, 253 (1968).
14. Comings, D. E., and E. Mattoccia, Proc. Nat. Acad. Sci., U.S., 67, 448 (1970).
15. Smith, B. J., J. Mol. Biol., 47, 101 (1970).