Chromatin maturation depends on continued DNA-replication

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The structure of [^{3}H]thymidine pulse-labeled chromatin in lymphocytes differs from that of non-replicating chromatin by several operational criteria which are related to the higher nuclease sensitivity of replicating chromatin. These structural features of replicating chromatin rapidly disappear when the [^{3}H]thymidine pulse is followed by a chase in the presence of an excess of non-radioactive thymidine. However, when the rate of DNA replication is reduced, as in cycloheximide-treated lymphocytes, chromatin maturation is retarded. No chromatin maturation is observed when nuclei from pulse-labeled lymphocytes are incubated in vitro in the absence of DNA precursors. In contrast, when these nuclei are incubated under conditions known to be optimal for DNA replication, the structure of replicating chromatin is efficiently converted to that of 'mature', non-replicating chromatin. We conclude that the properties of nascent DNA and/or the distance from the replication fork are important factors in chromatin maturation.

Chromatin maturation *DNA replication* *Lymphocyte* *Cycloheximide* *Micrococcal nuclease*

1. INTRODUCTION

Nuclease digestion of cellular chromatin has shown that the structure of newly replicated ([^{3}H]thymidine pulse-labeled) chromatin differs from that of non-replicating chromatin by several operational criteria:

(i) Newly-replicated chromatin is more sensitive to nuclease attack than bulk chromatin [1–4];
(ii) Monomeric nucleosomes are released faster from newly-replicated chromatin than from bulk chromatin [3,5];
(iii) During nuclease treatment, a large fraction of newly-replicated chromatin yields DNA fragments that are much shorter (55 ± 15 bp, 'subnucleosomal DNA') than those on standard nucleosomes (165 ± 3 bp, 'nucleosomal DNA' [4,7]). 'Subnucleosomal' DNA is associated with nucleosome-like chromatin fragments although in a way that makes this DNA–protein interaction sensitive against salt concentrations (0.5 M NaCl) which are insufficient to dissociate nucleosomal DNA from nucleosomes [7,8].

Upon progression of the replication fork, chromatin maturation occurs and [^{3}H]thymidine-labeled chromatin acquires a structure indistinguishable from that of non-replicating bulk chromatin.

We show here that chromatin maturation depends on continued DNA replication. We also demonstrate a strong influence of inhibitors of protein synthesis on the rate of chromatin maturation.

2. MATERIALS AND METHODS

The preparation and cultivation of bovine lymphocytes have been described in [9]. If required, concanavalin A-activated lymphocytes were continuously labeled with 1 μCi/ml [^{14}C]thymidine for 12–16 h. In some experiments, 50 μg/ml cycloheximide was added to the activated lymphocyte culture. This treatment reduces the rate of

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[^{3}H]leucine incorporation to 5–15% of a control culture. Incorporation of [^{3}H]thymidine was reduced to 30–40%, as compared to an untreated control culture.

Lymphocytes in the S-phase of the cell cycle were pulse-labeled with [^{3}H]thymidine as follows. The cells were concentrated by centrifugation and resuspended in fresh, pre-warmed medium at $2–3 \times 10^7$ cells/ml. 0.15–0.25 mCi [^{3}H]thymidine was then added for the times indicated below. The pulse was terminated by a 1:100 dilution into ice-cold Hank's-balanced salt solution and rapid centrifugation. If the pulse was followed by a chase, the labeled cells were diluted 1:100 into pre-warmed medium, containing 2 μg/ml non-radioactive thymidine, and incubated for the desired time at 37°C.

Nuclei were prepared as in [10,11]. The nuclei were incubated for in vitro DNA replication with ATP and a mixture of deoxyribonuclease triphosphates, including [^{32}P]dTTP, as in [11].

Micrococcal nuclease (Boehringer, Mannheim) was used for nucleolytic digestion of chromatin in intact nuclei. Details of the procedure and the analysis of the digestion products were as described in [4,7,11].

For the production of oligomeric groups of nucleosomes, washed nuclei (6×10^7) were treated in nuclease buffer [4] for 40–120 s at 37°C with 10 units micrococcal nuclease. Nuclear lysates were analyzed by centrifugation through linear 10–30% sucrose gradient, made up in lysis buffer, using the Beckman SW40 rotor for 3.5 h at 38000 rev./min and 7°C. After centrifugation, the gradient was pumped through a quartz cuvette to monitor the absorbance at 258 nm. The distribution of radioactivity was determined in the gradient fractions by trichloroacetic acid precipitation after treatment with 2 M NaOH for 1.5 h at 60°C.

3. RESULTS

3.1. *Chromatin maturation in the presence of cycloheximide*

Lymphocytes were long term-labeled with [^{14}C]thymidine and pulse-labeled for 30 s with [^{3}H]thymidine. A part of the culture was used immediately for nuclei preparation. Another part was chased with an excess of unlabeled thymidine. The chase was performed in the absence and, in a parallel experiment, in the presence of cycloheximide. Nuclei, prepared after the pulse and after the chase periods of various lengths, were briefly treated with micrococcal nuclease to obtain oligomeric nucleosome complexes. The soluble digestion products were investigated by sucrose gradient centrifugation (fig.1). The digestion products of [^{3}H]pulse-labeled chromatin sedimented mostly like monomeric nucleosomal particles (fig.1A) while most of the soluble bulk chromatin fragments were clearly much larger and may correspond to oligomeric complexes of 4, 5, 6 and more nucleosomes. After a 5 min chase in the absence of cycloheximide, substantial amounts of [^{3}H]thymidine-labeled nucleosomes appeared in fractions with higher order chromatin complexes (fig.1B). The maturation process was virtually complete after a 15 min chase (fig.1C). In the presence of cycloheximide, however, most radioactively-labeled chromatin fragments still sedimented like monomeric nucleosomes after 5 and 15 min chase periods (fig.1D,E). Even after a 60 min chase (fig.1F) most of the [^{3}H]thymidine-labeled chromatin fragments sedimented slower than most ^{14}C-labeled bulk chromatin.

Thus, maturation of replicating chromatin is inhibited in cycloheximide-treated cells. This could be due to a depletion of the histone pools and a reduced synthesis of new histones. But it is also possible that the retardation of DNA replication, a typical cycloheximide effect ([12,13]; see section 2), influences the maturation process. This possibility will be considered below.

3.2. *DNA synthesis and chromatin maturation*

We used isolated nuclei to study the effect of DNA replication on chromatin maturation. In agreement with others [10,14], we have shown that isolated lymphocyte nuclei perform semiconservative DNA replication when incubated under proper conditions with ATP and the 4 deoxynucleoside triphosphates, and that in vitro replicated DNA is organized in nucleosomal particles [8,11].

In a first experiment, nuclei were prepared from [^{14}C]thymidine continuously-labeled and [^{3}H]thymidine pulse-labeled (30 s) lymphocytes. The nuclei preparation was divided into 2 equal fractions. One fraction was incubated with ATP and an optimal mixture of all 4 deoxynucleoside

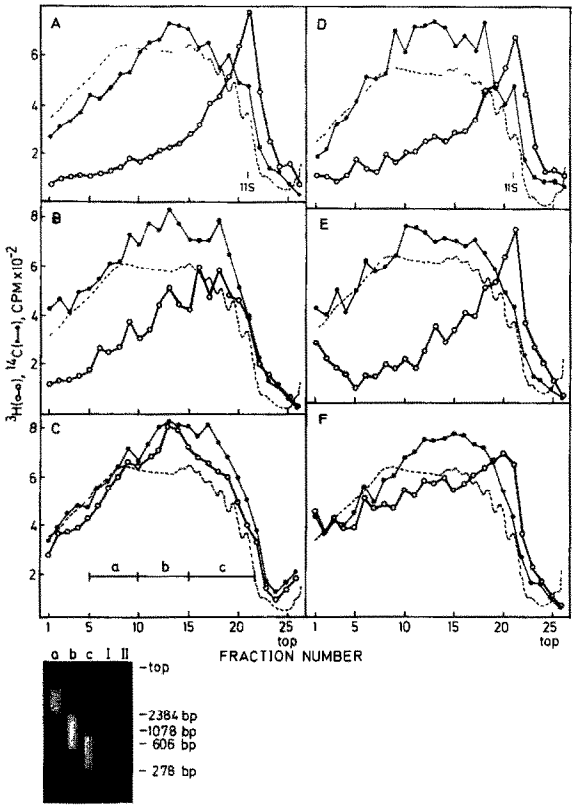

Fig.1. Chromatin maturation in vivo. Concanavalin A-activated lymphocytes were labeled for 16 h with [^{14}C]thymidine and pulse-labeled for 30 s with [^{3}H]thymidine. A fraction of the pulse-labeled cells was collected at 0°C by centrifugation (A). The remaining culture was split into 5 parts: one part was chased in the presence of an excess of non-radioactive thymidine for 5 min (B); another was chased for 15 min (C). The 3 additional parts received 50 μg/ml cycloheximide and were then chased for 5 min (D), 15 min (E) and 60 min (F), respectively. Nuclei (6×10^7) from these samples were briefly treated with micrococcal nuclease and lysed in the presence of EDTA. Soluble material was analyzed by sucrose gradient centrifugation. (– – –) Absorbance at 258 nm; (●) ^{14}C-cpm/fraction; (○) ^{3}H-cpm/fraction. Six fractions from 3 consecutive sections of one gradient were combined as indicated in panel C. The DNA was extracted from each pool in 0.1% sodium dodecylsulfate. The samples were treated with RNase and pronase, followed by phenol–chloroform extraction and ethanol precipitation. The extracted DNA was analyzed by electrophoresis in a 1% agarose gel. *Hae*II-restricted ColE1-DNA (I) and *Hae*III-restricted phage ϕX 174-RF-DNA (II) served as size markers.

triphosphates. The second fraction of the nuclei preparation was incubated under the same conditions except that nucleotides were omitted.

After incubation, the nuclei were transferred into nuclease buffer and treated with micrococcal nuclease as indicated in fig.2. A part of a 5 min-nuclease digest was used for sucrose gradient centrifugation in the presence of 0.5 M NaCl.

The digestion kinetics of radioactive chromatin from nuclei, incubated without nucleotides (fig.2A), was identical to that observed for freshly prepared pulse-labeled nuclei (not shown) indicating that a reorganization of chromatin had not occurred during the in vitro incubation period. In freshly prepared nuclei and in nuclei incubated in the absence of DNA synthesis, we found (fig.2A) that pulse-labeled chromatin is clearly more rapidly degraded than continuously-labeled chromatin. We also found that 'subnucleosomal' 4–5 S DNA appeared when the micrococcal digestion products were centrifuged through a sucrose gradient, containing 0.5 M NaCl (fig.2B). We have shown before [7,8] that [^{3}H]thymidine-labeled subnucleosomal 4–5 S DNA of 55 ± 15 bp length (see insert in fig.2B) is released in 0.5 M NaCl from chromatin fragments containing newly-replicated DNA. As mentioned in section 1, this property may be related to a conformational transition in nucleosomes passed from parental chromatin to newly-replicated DNA at the fork. (In the experiment of fig.2B, only a relatively small fraction of pulse-labeled chromatin had the peculiar structure which gives rise to subnucleosomal DNA fragments after brief nuclease treatment. The fraction of ^{3}H-labeled 4–5 S DNA is inversely related to the length of the pulse time [8]. It is therefore possible that, in the experiment of fig.2B, some chromatin maturation occurred already in vivo and/or during the preparation of nuclei.)

The structure of chromatin appears to be quite different when the nuclei of pulse-labeled cells were incubated in the presence of ATP and deoxynucleotides:

(i) The digestion kinetics of ^{3}H-labeled chromatin was very similar to that of ^{14}C continuously-labeled chromatin (fig.2C);

(ii) After centrifugation in the presence of 0.5 M NaCl, very little 4–5 S 'subnucleosomal' DNA was detectable.

The observations, shown in fig.2, demonstrate

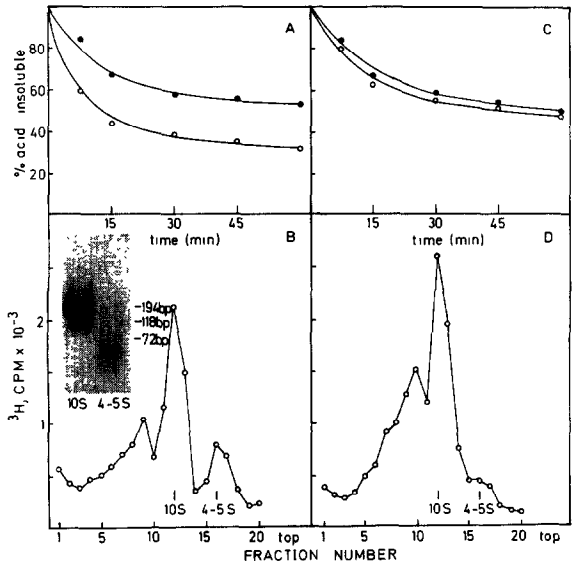

Fig.2. DNA replication and chromatin maturation. A lymphocyte culture was long term-labeled with [^{14}C]thymidine and pulse-labeled for 30 s with [^{3}H]thymidine. Nuclei were prepared and incubated in two equal aliquots in a buffer system, optimal for in vitro DNA replication [11]. (A), (B) One aliquot was incubated at 30°C for 40 min without nucleotides. (C), (D) The second aliquot received optimal concentrations of ATP and the 4 standard deoxynucleoside triphosphates, dATP, dGTP, dCTP and dTTP [11]. This sample was also incubated at 30°C for 40 min. (A), (C) After incubation the nuclei were treated with micrococcal nuclease (45 units/3 × 10^7 nuclei), and equal samples were removed at the indicated times to determine the radioactivity which remains precipitable by trichloroacetic acid [4]: (●) ^{14}C-cpm; (○) ^{3}H-cpm. (B), (D) Aliquots were removed after 10 min from the nuclease digestion mixtures, shown in (A) and (C), lysed in the presence of EDTA and centrifuged through sucrose gradients, containing 0.5 M NaCl. The distribution of acid-precipitable ^{3}H-radioactivity (○) is shown. Insert: in an experiment, performed under identical conditions, the fractions, labeled 10 S and 4–5 S, were separately combined and used for DNA extraction. Equal amounts of the ^{3}H-labeled DNA were investigated by polyacrylamide gel electrophoresis (fig.1, [7]) and fluorography. The DNA size markers are from *Hin*dIII-digested φX 174-RF-DNA.

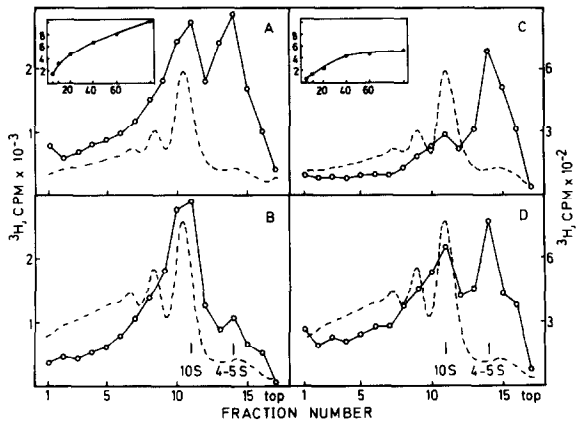

Fig.3. DNA replication and chromatin maturation in isolated nuclei. In vitro pulse and chase. Nuclei were prepared from a normal (A,B) and from a cycloheximide-treated (C,D) lymphocyte culture. Aliquots were incubated under DNA replication conditions [11] with ATP and deoxyribonucleoside triphosphates, including [^{3}H]dTTP, to determine the DNA replicating capacity of the nuclei preparations (insert in A and C: ordinates, incorporated [^{3}H]-cpm × 1000; abscissa, time of incubation, min at 30°C). Other aliquots of the nuclei preparations were incubated for 45 s under DNA replication conditions, using [^{3}H]dTTP in a specific radioactivity 10-times higher than that used in the experiments, shown in the inserts. One-half of these aliquots were removed after the 45 s-incubation (A,C). A 100-fold excess of non-radioactive dTTP was added to the second half, and the incubation was continued for another 20 min at 30°C (B,D). The nuclei were then digested with micrococcal nuclease until about 20% of the DNA was acid-soluble. Soluble chromatin fragments were centrifuged through sucrose gradients, containing 0.5 M NaCl. (– – –) Absorbance at 258 nm; (○) ^{3}H-cpm/fraction.

that maturation of newly-replicated chromatin depends on active DNA replication.

In fig.3 we present an experiment to support this conclusion and to demonstrate that pre-treatment of lymphocytes with cycloheximide severely inhibits chromatin maturation in isolated nuclei. In this experiment, nuclei were prepared from an S-phase lymphocyte culture (fig.3A,B) as well as from lymphocytes, treated during the S-phase with cycloheximide for 30 min (fig.3C,D). The isolated nuclei were incubated under standard conditions [11] with ATP and deoxyribonucleoside triphosphates, including [^{3}H]dTTP to allow for DNA replication in vitro. After 45 s, one half of each nuclei preparation was quickly transferred to 10 vol. ice-cold nuclease buffer (fig.3A,C). The second portion was further incubated for 20 min in the presence of a 100-fold excess of non-radioactive dTTP ('chase') before the reaction was

stopped in cold nuclease buffer (fig.3B,D). The nuclei were treated with micrococcal nuclease until about 20% of the DNA became soluble. The chromatin fragments were analyzed by gradient centrifugation in the presence of 0.5 M NaCl. We found a large fraction of in vitro pulse-labeled DNA as 4–5 S 'subnucleosomal' DNA (fig.3A,C). After a 20 min chase, most of this fraction disappeared in nuclei from normal, but not in nuclei from cycloheximide-pretreated cells in which only ~50% of the 4–5 S DNA could be chased into nucleosomal DNA (fig.3B,D). This may in part be due to the reduced DNA replication capacity of cycloheximide nuclei (compare inserts in fig.3A,C). It is also possible that the cycloheximide nuclei lack proteins, including histones, required for chromatin maturation.

4. DISCUSSION

We have investigated the structure of replicating chromatin, pulse-labeled in vivo with [^{3}H]thymidine, by a brief treatment with micrococcal nuclease, followed by sucrose gradient analysis of the nuclease degradation products. Under our experimental conditions, we found most soluble bulk chromatin in degradation products sedimenting with 30 S or more. Most of these degradation products contain DNA fragments of more than 2000 nucleotide pairs, sufficient to carry 8 or more individual nucleosomes. These supranucleosomal structures could be organized in higher order chromatin structures, such as solenoids [15]. In contrast, pulse-labeled DNA, when investigated in the same experiment, appears mainly on monomeric nucleosomes. This result is in agreement with earlier experiments [4,6] which demonstrated that one structural feature of newly-replicated chromatin can be operationally defined by the fast rate of nucleolytic cleavages between those nucleosomes which contain pulse-labeled DNA.

Several lines of evidence suggest that this peculiar structure of newly-replicated chromatin may be due to a lack of histone H1 on newly-replicated chromatin:

(i) Monomeric nucleosomes are produced significantly faster during nuclease treatment of H1-depleted chromatin than during nuclease treatment of native chromatin [16,17];
(ii) Monomeric nucleosomes, produced under mild digestion conditions of native chromatin, contain reduced amounts of H1, and the amount of chromatin-associated H1 increases with increasing size of chromatin fragments [18];
(iii) Monomeric nucleosomes, containing pulse-labeled DNA, travel in electrophoresis gels like H1- (and HMG-) depleted nucleosomes [7].

Since histone H1 is assumed to play an essential role in the maintenance of higher order chromatin structure [15,19] it is tempting to speculate that histone H1 is released from, or more weakly bound to, chromatin in the vicinity of replication forks. This would lead to a more open structure of replicating chromatin, and consequently, a facilitated access of replication factors to DNA. Our experiments have shown that, during a 15-min chase period in vivo, most pulse-labeled chromatin assumes the structure of bulk chromatin. This behavior is in agreement with earlier observations which showed that the association of histone H1 is the latest step in the assembly of chromatin [20].

In the presence of cycloheximide, the maturation process proceeds at a reduced rate. The reason for this is not entirely clear. One possible reason is certainly the depletion of histone pools and the inhibition of histone synthesis in cycloheximide-treated cells. We investigate, however, in our experiments, not only the structure of chromatin which is newly-assembled on freshly-replicated DNA but also the 50% fraction of chromatin which retains parental nucleosomes. Substantial evidence has accumulated to demonstrate that nucleosomes pass as conserved and intact entities [21–23] from the parental DNA stem to one [3,23,24] or both [25] replicated DNA branches. It is not obvious why the maturation of that chromatin fraction which retains the set of parental nucleosomes should be delayed in cycloheximide-treated cells. This could be due to a requirement for the continuous synthesis of a 'maturation factor'.

Another possibility is suggested by our experiments, presented in fig.2 and 3, which show that chromatin maturation depends on continued DNA replication. (Note that the rate of

[^{3}H]thymidine incorporation is reduced by 60–70% in cycloheximide-treated cells; see section 2 and [12,13].) We have tested two parameters of chromatin structure in nuclei from [^{3}H]thymidine pulse-labeled cells, the enhanced nuclease sensitivity as an indication of an extended chromatin structure and of histone depletion as well as the appearance of 4–5 S subnucleosomal DNA as an indication of an altered nucleosome structure. We found a reversion to the normal nuclease sensitivity and to a normal nucleosome structure only in nuclei, incubated under DNA replication conditions. When DNA replication was not possible due to a lack of DNA precursors and ATP the maturation process was impaired (fig.2).

This observation suggests that the structure of replicating DNA and/or the distance from the replication fork are important factors in chromatin maturation [1,6]. If replication points were fixed to attachment sites on the nuclear matrix, as proposed in [27–29], chromatin maturation could be prevented for spatial reasons; e.g., when replicating chromatin is so closely associated with matrix proteins that its folding to form higher order structures is sterically inhibited.

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REFERENCES

[1] DePamphilis, M.L. and Wassarman, P.M. (1980) Annu. Rev. Biochem. 49, 627–666.
[2] Seale, R.L. (1975) Nature 255, 247–249.
[3] Seale, R.L. (1976) Cell 9, 423–429.
[4] Schlaeger, E.J. and Klempnauer, K.H. (1978) Eur. J. Biochem. 89, 567–574.
[5] Hildebrand, C.E. and Walters, R.A. (1976) Biochem. Biophys. Res. Commun. 73, 157–163.
[6] Klempnauer, K.H., Fanning, E., Otto, B. and Knippers, R. (1980) J. Mol. Biol. 136, 359–374.
[7] Schlaeger, E.J. (1982) Biochemistry 21, 3167–3174.
[8] Schlaeger, E.J. and Knippers, R. (1979) Nucleic Acids Res. 6, 645–656.
[9] Peters, J.H. (1975) Methods Cell Biol. 9, 1–11.
[10] Benz, W.C. and Strominger, J.L. (1975) Proc. Natl. Acad. Sci. USA 72, 2413–2417.
[11] Schlaeger, E.J. (1978) Biochem. Biophys. Res. Commun. 81, 8–18.
[12] Stimac, E., Housman, D. and Huberman, J.A. (1977) J. Mol. Biol. 115, 485–511.
[13] Seale, R.L. and Simpson, R.T. (1975) J. Mol. Biol. 94, 479–501.
[14] Sheinin, R., Humbert, J. and Pearlman, R.E. (1978) Annu. Rev. Biochem. 47, 277–316.
[15] Thoma, F., Koller, T. and Klug, A. (1979) Cell Biol. 83, 403–427.
[16] Noll, M. and Kornberg, R. (1977) J. Mol. Biol. 109, 393–404.
[17] Weischet, W.O., Allen, J.R., Riedel, G. and Van Holde, K.E. (1979) Nucleic Acids Res. 6, 1843–1861.
[18] Renz, M., Nehls, P. and Hozier, J. (1977) Proc. Natl. Acad. Sci. USA 74, 1879–1883.
[19] Müller, U., Zentgraf, H., Eicken, I. and Keller, W. (1978) Science 201, 406–415.
[20] Worcel, A., Han, S. and Wong, M.L. (1978) Cell 15, 969–977.
[21] Seale, R.L. (1976) Cell 9, 423–429.
[22] Leffak, J.M., Grainger, R. and Weintraub, H. (1977) Cell 12, 837–845.
[23] Seidman, M.M., Levine, A.J. and Weintraub, H. (1979) Cell 18, 439–449.
[24] Riley, D. and Weintraub, H. (1979) Proc. Natl. Acad. Sci. USA 76, 328–332.
[25] Jackson, V., Grauner, D. and Chalkley, R. (1976) Proc. Natl. Acad. Sci. USA 73, 2266–2269.
[26] Berezney, R. and Coffey, D.S. (1975) Science 189, 291–293.
[27] Dijkwel, P.A., Mullenders, L.H.F. and Wanka, F. (1979) Nucleic Acids Res. 6, 219–230.
[28] Pardoll, D.M., Vogelstein, B. and Coffey, D.S. (1980) Cell 19, 527–536.