Purification of 14C-Labelled Deoxyribonuclease II from HeLa S3
Lysosomes and its Use as a Marker for the Study of Nuclear
Deoxyribonuclease II

By HANOCH SLOR*
Department of Human Genetics, Tel Aviv University Medical School, Tel Aviv, Israel
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Deoxyribonuclease II (DNAase II) in mammalian cells has generally been considered to
be located in the lysosomes. Several recent studies have indicated that some DNAase II
activity is present in purified nuclei; this, however, could have been due to some contami-
nation of the nuclear fraction by lysosomes, or alternatively, it could have been caused by
specific binding of lysosomal DNAase II to the nuclear fraction during isolation. Our
previous studies have eliminated the possibility that lysosomal contamination was the
cause of the presence of DNAase II in isolated nuclei. In this study I have purified 14C-
labelled lysosomal DNAase II and added it to cells during isolation of their nuclei. This
study demonstrates that there is no specific binding of lysosomal DNAase II to the nuclear
fraction and concludes that DNAase II activity observed in isolated nuclei represents an
intrinsic activity that might be involved in nuclear DNA metabolism.

Studies of various DNAases† in both bacterial and
mammalian cells indicate that some may have an
important role in cellular DNA metabolism (DNA
repair, DNA recombination, DNA synthesis, DNA
degradation) (see reviews by Lehman, 1967, and by
Lesca, 1971). If so, they are most likely to be present
in the nucleus where most of the DNA is located.
Indirect support for this hypothesis came from
comparative studies of DNAases (Allfrey & Mirsky,
1952; Cordonnier & Bernardi, 1968), which suggested
a correlation between the activity of DNAase II
(acid DNAase) and the capacity of certain tissues to
divide. By using histochemical methods, Swingle &
Cole (1964) detected DNAase II activity in rat liver
nuclei directly. Lesca (1968) also demonstrated the
enzyme’s presence in the nuclear fraction of mouse
liver cells. Assaying the nuclear fraction for cyto-
chrome oxidase, which is absent from the nucleus
(de Duve et al., 1962), Lesca (1968) concluded that
the nuclear DNAase II activity was not due to
lysosomal contamination. We have recently demon-
strated the presence of DNAase II activity in purified
calf thymus nuclei by using acid phosphatase as a
marker for lysosomal or cytoplasmic contamination
of the nuclear fraction (Slor & Lev, 1971). However,
the use of cytoplasmic enzymes as a measure of purity
of the nuclear fraction cannot eliminate the possibility
of preferential binding of cytoplasmic DNAase II to
the nuclear DNA during preparation of the nuclei.
The purpose of the present work was to test whether

* Present address: Laboratory of Radiobiology,
University of California, San Francisco, Calif. 94122,
U.S.A.
† Abbreviation: DNAase, deoxyribonuclease.

the finding of DNAase II activity in the nucleus was
due to preferential binding of lysosomal DNAase II
to the nuclei during the isolation of the nuclear frac-
tion, or if it represents an intrinsic nuclear DNAase II
activity. The basic approach to this problem was to
isolate lysosomes from HeLa S3 cells prelabelled
with radioactive amino acid to label DNAase II
molecules and to purify this enzyme to relatively high
purity. An excess of such purified enzyme added to
cells during cell disruption, and isolation of their
nuclei, should indicate any preferential DNAase II
binding to nuclei by following the binding of acid-
insoluble radioactivity into the purified nuclei.

Materials and Methods

HeLa S3 cells were grown as monolayers and
maintained in equal parts of Eagle’s minimum
essential medium and M199 medium supplemented
with 10% (v/v) calf serum and antibiotics (200 i.u.
penicillin/ml and 20 i.u. of dihydrostreptomycin/ml)
and with 0.5 ml of 3% glutamine/100 ml, as described
by Slor et al. (1973). To label the nuclei, cells in the
exponential growth phase were incubated with 1μCi
of [3H]thymidine/ml of medium (methyl-3H)thymi-
dine, 3Ci/mmole, Schwarz/Mann) for 60 min. The
radioactive medium was then replaced and the cells
were allowed to grow in the 3H-free medium for
6 h more, before being collected by trypsinization
(DNAase II-free trypsin) and washed three times with
0.05M-sodium phosphate-buffered saline (PBS),
PH 7. To these cells the purified [14C]DNAase II was
added (details in the text), cells were disrupted by
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sonication in an iced-water bath (MSE sonicator, 30 s, 8 μm between peaks).

For labelling DNAase II with $^{14}$C, HeLa S3 cells were grown in Eagle's minimal essential medium without leucine and after 4–5 h, 5 μCi of $^{[14]C}$leucine/ml of medium (pH-2–14]Cleucine, 48 mCi/mmol, Schwarz/ Mann) was then added and the cells were incubated with the label for 70 h. Cells were then detached by trypsinization (DNAase II-free trypsin) and washed three times with PBS medium. Cells resuspended in 15 ml of 0.25 M-sucrose–0.01 M-Tris–HCl, pH 7–0.01 M-MgCl$_2$ ($2 \times 10^7$ cells/ml) were then homogenized for 2 min in a Potter–Elvehjem (Teflon pestle) homogenizer. Nuclei were removed by centrifugation at 700 g for 10 min. The supernatant was centrifuged at 10000 g for 1 h at 4°C. The pellet was washed with 5 ml of the same buffer and re-centrifuged again as above. The pellet contained both lysosomes and mitochondria as determined by assaying acid phosphatase (Slor & Lev, 1971) as a lysosomal marker and cytochrome oxidase as a mitochondrial marker (Appleman et al., 1955). Approximately 70% of total cellular acid phosphatase and 80% of cellular cytochrome oxidase was recovered in this fraction. Some preparations had as much as 85% lysosomal recovery by this procedure. The lysosomes were further purified as follows. The lysosome–mitochondria pellet was resuspended in 5 ml of 8% sucrose (buffered with 0.05 M-potassium phosphate, pH 7) and was layered on top of a gradient tube containing layers of 4 ml each of 30, 25, 15 and 10% (w/v) sucrose (buffered as above) in a 50 ml polycarbonate centrifuge tube. Centrifugation was at 1350 g for 120 min at 4°C. Fractions of 1.6 ml were collected from the bottom of the tube (16 fractions). A sample from each tube was sonicated and assayed for both acid phosphatase and cytochrome oxidase activity. The acid phosphatase peak was at tube 10 whereas the cytochrome oxidase peak was at the top of the gradient (tube 16) with a rather long shoulder reaching to fraction 11. The pooled lysosomal fractions (tubes 7–12) contained about 45–70% of total cellular acid phosphatase activity (the yield varied in different preparations) and less than 5% of the total cellular cytochrome oxidase activity. The purified lysosomal preparation used to purify the $^{14}$C-labelled DNAase II came from a preparation with a lysosomal yield of 65%.

To purify the $^{14}$C-labelled lysosomal DNAase II, the purified lysosomes were sonicated in their sucrose medium and dialysed for 24 h against 20 vol. of 0.05 M-sodium phosphate buffer, pH 6.5, with one change of dialysis buffer. The retentate was centrifuged for 60 min at 15000 g at 4°C and the supernatant was applied to a CM-cellulose column (0.9 cm × 10 cm) that had previously been equilibrated with the dialysis buffer. The column was washed with the same buffer to remove proteins not bound to the resin. No DNAase II came off the column at this step. The enzyme was eluted from the CM-cellulose by a linear gradient of 0.05 M- to 0.25 M-sodium phosphate buffer, pH 6.5. Fractions of 1.5 ml were collected at a flow rate of 20 ml/h. Tubes were assayed for DNAase II activity, protein content (by the method of Lowry et al., 1951, with bovine serum albumin as standard) and acid-insoluble $^{14}$C (Fig. 1). Tubes 21–28, which contained most of the DNAase II activity, were pooled (CM-cellulose pool) and (NH$_4$)$_2$SO$_4$ was

![Fig. 1. Purification of DNAase II labelled with $^{14}$C]leucine on CM-cellulose](image)

The column (0.6 cm × 9 cm) was equilibrated with 0.05 M-sodium phosphate buffer, pH 6.5, and loaded with retentate from $3 \times 10^8$ cells containing 25000 DNAase II units with a specific activity of 2083. The enzyme was eluted by a linear gradient of 0.05 M- to 0.25 M-sodium phosphate buffer, pH 6.5, in a total volume of 60 ml. Fractions of 1.5 ml were collected at a flow rate of 20 ml/h. (●) DNAase II activity (units/fraction); (○) protein content; (▲) acid-insoluble $^{14}$C.
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added to 50% saturation. After the sediment was removed by centrifugation, more (NH₄)₂SO₄ was added to 75% saturation and the preparation was left in ice for several h. The sediment was collected by centrifugation, dissolved in 1.5ml of 0.05M-sodium phosphate buffer, pH 6.5, and layered on a Sephadex G-75 column (0.8cm × 18cm) equilibrated with the same buffer. The enzyme was eluted with the same buffer (Fig. 2) at a flow rate of 20ml/h. Fractions that contained DNAase II activity were pooled (Sephadex G-75 pool, tubes 5–7) and the specific activity (units/mg of protein) determined. The specific activity of the enzyme in the pooled fractions was 1.27 × 10⁶ and that of tube 6 was 2.42 × 10⁶, 500 and 1000 times higher than that in the crude extract (Table I).

Nuclei from ³²P-labelled HeLa cells were prepared in the presence of purified lysosomal ¹⁴C-labelled DNAase II. ¹⁴C-labelled lysosomal DNAase II (15000 units) was added to 2 × 10⁸ cells (containing a total of 1850 units of DNAase II) suspended in 0.25M-sucrose – 0.01M-Tris-HCl, pH 7.0.01M-MgCl₂ (2ml), and homogenized for 2min in a Potter–Elvehjem homogenizer. The pellet was washed three times with 1ml of the same buffer and the nuclei were resuspended in 8ml of 2.2M-sucrose – 5mM-MgCl₂ – 1mM-potassium phosphate, pH 6.8, and centrifuged in a Beckman Spincor L2 preparative ultracentrifuge at 25000rev./min (approx. 70000g) at 4°C in a No. 40 rotor. After 60min the supernatant was carefully removed and the pellet was resuspended in 2ml of 0.25M-sucrose – 1mM-MgCl₂ – 2mM-potassium phosphate, pH 6.8, and centrifuged at 1200g for 10min at 4°C. The nuclear pellet was then washed for 5min in 0.14M-NaCl – 1mM-MgCl₂ – 2mM-potassium phosphate, pH 6.4, to which was added 0.5% of Triton X-100. This treatment facilitated resuspension of the nuclei. Nuclei were then collected by centrifugation at 1200g as described above and were used for the determination of DNAase II activity and of the acid-insoluble radioactivity of both ³²P-labelled DNA and ¹⁴C-labelled lysosomal DNAase II. The procedure used for the isolation of the nuclei is a combination of the procedures used by Kapp & Okada (1972) and by Laval & Bouteille (1973). The latter authors found

Table 1. Purification of ¹⁴C-labelled DNAase II from lysosomes of HeLa S3 cells

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (µg)</th>
<th>Total DNAase II activity (units)</th>
<th>10⁻⁳ × Specific activity (units/mg)</th>
<th>Total acid-insoluble ¹⁴C (c.p.m.)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract (from 3 × 10⁸ cells)</td>
<td>13200</td>
<td>30000</td>
<td>2.27</td>
<td>1.95 × 10⁴</td>
<td>0</td>
</tr>
<tr>
<td>Diffusate</td>
<td>12000</td>
<td>25000</td>
<td>2.08</td>
<td>1.8 × 10⁵</td>
<td>0</td>
</tr>
<tr>
<td>CM-cellulose pool</td>
<td>370</td>
<td>21700</td>
<td>58.6</td>
<td>5.4 × 10⁶</td>
<td>25.8</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (50–75% satn.)</td>
<td>100</td>
<td>18500</td>
<td>185</td>
<td>2.1 × 10⁶</td>
<td>81.5</td>
</tr>
<tr>
<td>Sephadex G-75 pool</td>
<td>12</td>
<td>15200</td>
<td>1267</td>
<td>1.9 × 10⁵</td>
<td>558</td>
</tr>
<tr>
<td>Sephadex G-75 peak fraction, tube 6</td>
<td>3.4</td>
<td>8300</td>
<td>2420</td>
<td>5.0 × 10⁴</td>
<td>1075</td>
</tr>
</tbody>
</table>

Fig. 2. Purification of ¹⁴C-labelled DNAase II on Sephadex G-75

A column (0.8cm × 18cm) was pre-equilibrated with 0.05M-sodium phosphate buffer, pH 6.5, and 0.5ml of the DNAase II (after the 50–75% (NH₄)₂SO₄ step) was layered on the column and eluted by the same buffer at a flow rate of 20ml/h. Fractions of 1ml were collected and assayed for DNAase II activity (○), for protein content (●) and for acid-insoluble ¹⁴C (▲).
that this procedure yields a very pure (and biologically active) nuclear preparation and that the detergent (Triton X-100) addition removes the outer nuclear membrane. As pointed out by Holtzman et al. (1966) detergents not only remove the outer nuclear membrane but also decrease the cytoplasmic contamination as a whole.

DNAase II was assayed as described by Slor & Hodes (1970) with the addition of 5 mM-EDTA to the reaction mixture. These conditions completely inhibit the activity of DNAase I, which otherwise might be slightly active at the pH of 4.5 used for the assay.

Acid-insoluble radioactivity [at a final concentration of 7% (w/v) cold trichloroacetic acid] was determined on GF/A glass-filter discs (Whatman). Filters were dried to evaporate the trichloroacetic acid and counted for radioactivity in a toluene-based scintillation fluid in a Packard Tri-Carb scintillation-spectrometer.

Results

\[ \text{Results} \]

\[ \text{14C-labelled lysosomal DNAase II was added to HeLa S3 cells that were pre-labelled with [3H]thymi-} \]

dine as a measure of the nuclear fraction. The cells were disrupted by homogenization in a Potter–Elvehjem homogenizer and the nuclei were isolated and purified as described in the Materials and Methods section. To ensure detection of any cytoplasmic DNAase II bound to the nuclear fraction during preparation of nuclei, the amount of purified \[ \text{14C-labelled lysosomal DNAase II} \]

Table 2. Separation of nuclear DNAase II from \[ [3H]thymidine-labelled HeLa S3 cells in the presence of purified \[ 14C-labelled lysosomal DNAase II \]

The results are the average of two experiments. \[ \text{14C-labelled purified lysosomal DNAase II (15000 units,} \]

1.9 \times 10^8\ c.p.m.) was added to 2 \times 10^7 \text{ HeLa S3 cells in 2 ml of 0.25 M-sucrose–0.01 M-Tris–HCl, pH 7–0.01 M-MgCl}_2, \text{ and homogenized for 2 min in a Potter–Elvehjem homogenizer (step 1). The homogenate was centrifuged at 700 g for 10 min at 4°C (step 2) and contained mainly nuclei and cell debris. The supernatant (step 3) was discarded. The pellet was washed three more times with the same buffer and the final pellet was resuspended in 8 ml of 2.2 M-sucrose–1 mM-potassium phosphate, pH 6.8–5 mM-MgCl}_2, \text{ and centrifuged in the Spinco L2 (rotor No. 40) at 4°C for 60 min. The supernatant was removed and the sediment (step 5) resuspended in 2 ml of 0.25 M-sucrose–1 mM-MgCl}_2–2 mM-potassium phosphate, pH 6.8, and centrifuged at 1200 g for 10 min at 4°C. The nuclear pellet was washed with the same solution but the pH of the potassium phosphate was 6.4 and it contained 0.5% of Triton X-100. Purified nuclei were collected by centrifugation at 1200 g for 10 min at 4°C (step 6), resuspended in 1 ml of sodium acetate buffer, pH 4.5, sonicated for 30 s and used to determine DNAase II activity and acid-insoluble radioactivity of both \[ \text{3H-labelled DNA} \]

and \[ \text{14C-labelled lysosomal DNAase II} \]. In the control experiment nuclei were purified from HeLa S3 cells without the addition of \[ 14C-labelled DNAase II \] before cell homogenization (step 7).

<table>
<thead>
<tr>
<th>Step</th>
<th>Total DNAase II activity (units)</th>
<th>Total acid-insoluble [ [3H] \text{ (c.p.m.)} ]</th>
<th>Total acid-insoluble [ 14C \text{ (c.p.m.)} ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>Homogenate from 2 \times 10^7 cells</td>
<td>1850 (+15000 14C-labelled lysosomal DNAase II units)</td>
<td>6480</td>
</tr>
<tr>
<td>(2)</td>
<td>Sediment of centrifuged homogenate</td>
<td>310</td>
<td>6340</td>
</tr>
<tr>
<td>(3)</td>
<td>Supernatant of step (2)</td>
<td>15050</td>
<td>285</td>
</tr>
<tr>
<td>(4)</td>
<td>Washed sediment of step (2)</td>
<td>275</td>
<td>6170</td>
</tr>
<tr>
<td>(5)</td>
<td>Sediment after 2.2 M-sucrose step (70000g, 60 min)</td>
<td>205</td>
<td>5915</td>
</tr>
<tr>
<td>(6)</td>
<td>Purified nuclei (after isosmotic Triton X-100 treatment)</td>
<td>175</td>
<td>5810</td>
</tr>
<tr>
<td>(7)</td>
<td>Control experiment (purified nuclei after step (6) but without the addition of 14C-lysosomal DNAase II)</td>
<td>182</td>
<td>5890</td>
</tr>
</tbody>
</table>
somal DNAase II during the purification of the nuclear fraction did not affect its final nuclear DNAase II content. Further, if the 175 DNAase II units in the nuclei were due to contamination by lysosomal DNAase II, one would expect to observe approximately 19000 c.p.m. of \(^{14}C\) in the nuclear fraction, whereas only 150 c.p.m. were found. This clearly indicates that the nuclear DNAase II activity was not due to a cytoplasmic contaminant but is found within the nucleus proper.

**Discussion**

The localization of DNAase II in mammalian cells has been a controversial subject. Among many reports on its cellular localization, Brown *et al.* (1952) described its presence in the nucleus and Schneider & Hogeboom (1952) and Koerner & Sinheimer (1957) in the mitochondria. Bowers (1964) and de Duve *et al.* (1962) described it as an exclusively lysosomal enzyme and discounted the presence of DNAase II in other cell organelles on the grounds that they were contaminated by lysosomes. Lehman (1967) and Lesca (1971) have reviewed some of the arguments for the involvement of DNAases in cell division, DNA synthesis and replication, using examples from bacterial, mammalian and viral-induced DNAases. One would expect to find an enzyme that was directly involved in nuclear DNA metabolism localized in the nucleus. It might be present only in the nucleus, or it might be present in other parts of the cell as well, in which case the same enzyme may play different roles at different locations in the cell.

Although we do not have conclusive evidence that the DNAase II in the nucleus is identical to the lysosomal enzyme, it does have properties in common with it, such as its pH–activity curve, absence of dependence on Mg\(^{2+}\) ions, and inhibition by SO\(_4^{2–}\) ions and by tRNA.

All experiments on DNAase II (acid DNAase) were performed at pH values between 4.5 and 5.5. However, Slor & Lev (1972) have shown that highly purified acid DNAase can be active at neutral pH, given the appropriate conditions and they suggested that the term 'DNAase II' rather than 'acid DNAase' should be used for this enzyme. In contrast to its activity at acidic pH, DNAase II at neutral pH requires divalent cations for its activity. Thus, many experiments on DNAase I, which is active at neutral pH and requires divalent cations, could have assayed the neutral activity of DNAase II.

Under standard conditions the DNAase II in the nucleus is approximately 10% of that of the lysosomal fraction. Some preliminary studies in our laboratory (H. Slor & T. Lev, unpublished work) suggest that the equilibrium between lysosomal and nuclear activities may be altered by agents affecting DNA synthesis. We have also shown that there is a correlation between DNAase II activity and DNA synthesis in synchronized HeLa S3 cells (Slor *et al.*, 1973).

Our experiments suggest that DNAase II activities found in HeLa cell nuclei and probably in other tissues, such as mouse liver nuclei (Swingle & Cole, 1964), rat liver nuclei (Lesca, 1968) and calf thymus nuclei (Slor & Lev, 1971), are not due to contamination of the nuclei by lysosomal enzyme but are rather an intrinsic nuclear activity. This finding may support the assumption that DNAase II might be involved in cellular DNA metabolism in a way that is not yet clear. Our current knowledge about DNA metabolism in the cell would support the assumption that specific DNAases participate in DNA repair, in DNA recombination and in DNA replication. DNAases might also be involved in removing mis-synthesized fragments of DNA during DNA synthesis. Further studies are certainly needed to find out in what capacity nuclear DNAase II is involved in DNA metabolism, if at all.

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**References**


