Purification and Characterization of a Chemically Induced Epstein-Barr Virus-Associated Deoxyribonuclease

Guang-Yuh Hwang
Utah State University
PURIFICATION AND CHARACTERIZATION OF A CHEMICALLY INDUCED
EPSTEIN-BARR VIRUS-ASSOCIATED DEOXYRIBONUCLEASE

by

Guang-Yuh Hwang

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Guang-Yuh Hwang
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ABSTRACT

Purification and Characterization of a Chemically Induced Epstein-Barr Virus-Associated Deoxyribonuclease

by

Guang-Yuh Hwang, Master of Science
Utah State University, 1989

Major Professor: Joseph K.-K. Li
Department: Biology

Purification of Epstein-Barr virus-associated deoxyribonuclease (EBV-DNase) from Raji and P3HR-1 cells treated with 12-0-tetradecanoylphorbol-13-acetate and sodium butyrate was performed by sequential ion-exchange column chromatography and fast protein liquid chromatography. The enzyme activity, protein concentration, yield, specific activity, purification profiles, and polypeptide patterns by electrophoretic analysis in each column purification step were determined. The characteristics of the partially isolated EBV-DNase were demonstrated by the enzyme activity, DNA binding affinity, and inhibition by the nasopharyngeal carcinoma patient sera and rabbit polyclonal antibodies against the partially purified EBV-DNase. A nonisotopic assay was developed as a new method in detecting the nuclease. EBV-DNase was purified to homogeneity by FPLC. The molecular weight of the EBV-DNase was 58 KDa as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis, immunostaining, and radioimmunoprecipitation using nasopharyngeal carcinoma patient sera and rabbit polyclonal antibodies.

(46 pages)
INTRODUCTION

Epstein-Barr virus (EBV), a member of the human gamma-herpesvirus family, is a B-lymphocyte-transforming virus that causes infectious mononucleosis (IM) (15, 30). Furthermore, there is strong evidence that EBV, as a human carcinogenic agent, causes African Burkitt's lymphoma (BL) (13) and nasopharyngeal carcinoma (NPC) (22) within specific races of people. The association of EBV to these two cancers is supported by the evidence that EBV genomes are found in BL and NPC tumor tissues of infected patients (12). These two types of cancer also have high malignancy and mortality rates as follows: BL is an endemic malignancy affecting mainly children in East Africa and New Guinea, while NPC is the most common malignancy in Southeast Asia. No specific reason has been found for the susceptibility of these populations, but it appears to involve both genetic and environmental factors (36).

EBV, as do other herpesviruses, has a biological property of inducing latent and persistent infection to the mature B-lymphocytes. The EBV target cells are mainly terminally differentiated cells that have receptors for complement binding and synthesize all of the different classes of the immunoglobulin heavy and light chains (33). The virus enters the host cell by fusion with the cell membrane. Viral DNA may be expressed or exist intracellularly in an unexpressed state known as latency that can be chemically activated to express. Upon activation by inducing agents, the viral DNA is transcribed and replicated in the nucleus, and the viral mRNAs are translated in the cytoplasm. The translation products then return to the nucleus where the nucleocapsid is assembled. The capsids acquire an envelope from the outer lamella of the nuclear membrane during assembly. The EBV viral antigens expressed during the latent and replicative cycles include the Epstein-Barr nuclear antigen (EBNA), lymphocyte-determined membrane antigen (LYDMA), viral capsid antigen (VCA), membrane antigen (MA), and early antigens (EA). The EBNA is expressed in EBV-immortalized human
lymphocytes and, thus, may play an important role in immortalization (16). The LYDMA, demonstrated using in vitro tests of cell-mediated cytotoxicity, is distinguished from viral membrane antigens (MA) that are found on the surfaces of cells synthesizing virions and are also components of the virion envelope (34). The VCA is found within the cells which are producing viruses. Patients with NPC have high antibody titers to EBV-VCA. The major approach in the study of the epidemiology of EBV infections is detection of the antibody to EBV-VCA (17). The MA, which is found on the cytoplasmic membranes of the cells that are producing virions, is also responsible for eliciting virus-neutralizing antibodies (10). Early antigens are a group of nonstructural components whose synthesis does not require viral DNA synthesis. They can be synthesized when an infected lymphoid cell is spontaneously triggered into viral replication or when the viral replicative cycle is induced by exogenous stimuli such as phorbol esters or sodium butyrate (SB). The functions of the proteins represented in the early antigen complex are still unknown but are likely associated with the enzymes that are required for viral DNA replication (31).

Three virus-encoded enzymes, including DNA polymerase (DP) (20), thymidine kinase (TK) (11), and deoxyribonuclease (DNase) (8), involved in viral replication have been identified. The presence of serum antibody of the IgA type to EBV-VCA is the basis of a screening test for the detection of NPC. However, it is not sensitive enough to detect the early EBV infection. Patients with BL, IM, and NPC have elevated levels of antibody to early antigens, which appears to be evidence of ongoing viral infection. High-titer antibodies to the EBV-DNase have been detected and proved to be a sensitive approach for the medical diagnosis of the early detection of EBV infections (4, 5).

Although the complete genome of EBV has been cloned and sequenced, the relationship between the viral products and their respective genes is still unknown (18). In order to unravel the mystery of EBV latency and its induction, a more detailed understanding of the viral enzymes involved in DNA replication is required. The
characterization and identification of the chemically induced EBV-associated DNase have been partially determined with respect to substrate specificity, strong divalent cation requirement (Mg\(^{2+}\)), alkaline pH optimum (pH 8.3), and high salt resistance (8, 35, 9, 6). However, EBV-DNase has not been purified to homogeneity. Thus, reports on the determination of the molecular weight (M.W.) of the EBV-DNase are quite controversial (see Table 1). This conflict may be caused by the differential production of EBV-DNase(s) induced in different cell lines (20, 8, 32) or by the presence of an EBV-DNase-associated protein, such as EBV-DNA polymerase (7).

Table 1. Summary of the known molecular weight of EBV-DNase

<table>
<thead>
<tr>
<th>Inducing agents</th>
<th>Cell lines</th>
<th>M.W.</th>
<th>Methods of Determination</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>B95-8</td>
<td>60 KDa</td>
<td>SDS-PAGE(^a)</td>
<td>Clough, 1979. (8)</td>
</tr>
<tr>
<td>P(_3)HR-1</td>
<td>Raji</td>
<td>120 KDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>P(_3)HR-1</td>
<td></td>
<td></td>
<td>Tan et al., 1982. (35)</td>
</tr>
<tr>
<td>TPA/SB(^b)</td>
<td>Raji</td>
<td>4.0 S</td>
<td>Sedimentation</td>
<td>Ooka et al., 1984. (32)</td>
</tr>
<tr>
<td>SB</td>
<td>P(_3)HR-1</td>
<td>70 KDa</td>
<td>Gel filtration</td>
<td>Kallin et al., 1985. (20)</td>
</tr>
</tbody>
</table>

\(^a\) Principal peptide bands of 60 KDa and 120 KDa were present, as well as several bands of lower molecular weights in the range of 30 KDa.

\(^b\) TPA stands for 12-0-tetradecanoylphorbol-13-acetate and SB stands for sodium butyrate.
Procedures to purify EBV-DNase by sequential column chromatography have been described (8, 35, 9). Because of difficulties in propagating EBV in large amounts, as well as poor recovery and the instability of EBV-DNase, it is necessary to induce very large numbers of cells in order to obtain sufficient amounts of enzyme for purification. Raji and P3HR-1 (see Table 2) cells that are latently infected with EBV exhibit increased expression of EBV-DNase activity when treated with TPA (37) and SB (32). TPA is a diterpene ester, known as a tumor-promoting agent that induces the gene expression of oncogenic herpes viruses in persistently infected cells (38). SB is a differentiation inducer that has been found to increase the number of virus-producer cells to a dramatic extent in the EBV-carrying cell lines (25).

Table 2. TPA-induced EBV-transformed cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Origin</th>
<th>% cell stained specifically for viral antigen (VCA,EA)</th>
<th>control</th>
<th>5d TPA b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raji</td>
<td>African Burkitt's lymphoma (EBV-nonproducer)</td>
<td>&lt;0.01</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td>P3HR-1</td>
<td>African Burkitt's lymphoma (EBV-producer)</td>
<td>9.1</td>
<td>74.3</td>
<td></td>
</tr>
</tbody>
</table>

a The data was adapted from Zur Hausen et al., 1978 (38).

b The cells were stained with a human EA+ VCA+ serum (1:40 dilution) 5 days after the addition of 20 ng/ml of TPA.

The frequency and levels of antibody against EBV-DNase in NPC-patient sera are much higher than in sera of healthy individuals or individuals with IM or BL. Thus, use of purified EBV-DNase may aid in the early diagnosis of NPC. Furthermore, the
identification of the M.W. and amino acid sequence of purified EBV-DNase will be useful in the determination of the viral coding region as well as the molecular aspects of EBV.

Therefore, the objectives of this thesis were to purify the chemically induced EBV-DNase to homogeneity, to determine its M.W., to investigate some of the biochemical characteristics of the purified EBV-DNase, and to produce polyclonal anti-EBV-DNase sera for immunological determinations and subsequent experimentation.
MATERIALS AND METHODS

Chemical induction in Raji and P3HR-1 cell lines. Lymphocyte cell lines have been previously described (14). Raji (a Burkitt's tumor nonproducer B-cell line) and P3HR-1 (a Burkitt's lymphoma-derived cell line) cells were maintained between 5 x 10^5 to 1 x 10^6 cells/ml in RPMI-1640 (GIBCO, Grand island, NY) medium containing 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT). Raji and P3HR-1 cells were seeded at 10^6 cells per ml in culture medium. TPA (Sigma, St. Louis, MO) was added at a final concentration of 40 ng/ml. SB was added at a final concentration of 4 mM. The culture was incubated at 37°C for 64-72 hrs.

Preparation of Escherichia coli [³H]-DNA. Native double-stranded E. coli [³H]-DNA was prepared from the thymine-requiring strain. The bacteria were grown in 2 liters of a minimal medium containing 40 mM K₂HPO₄, 22 mM KH₂PO₄, 2 mM Na₃C₆H₅O₇, 0.4 mM MgSO₄. H₂O, 7 mM (NH₄)₂SO₄, 0.25% glucose (w/v), 2.5 μM unlabelled thymine, and 500 μCi of ³H-thymine (NEN, Boston, MA). [³H]-DNA was isolated by the procedure of Marmur (26). Isolated E. coli [³H]-DNA was used as substrate in EBV-DNase enzyme activity assays.

EBV-DNase assay. EBV-specific DNase enzyme activity was measured by the release of acid-soluble nucleotides from double-stranded [³H]-DNA by the method of Hoffman and Cheng (18). Briefly, each reaction solution contained 0.2 ml of 50 mM Tris (pH 8.3), 10 mM MgCl₂, 10 mM β-mercaptoethanol, and 1.2 μg of E. coli [³H]-DNA. The enzyme assay mixture contained 10 μl of enzyme preparation plus 190 μl of the reaction solution. The assay was incubated at 37°C for 40 min with constant shaking. Following incubation, calf thymus DNA (Sigma, St. Louis, MO) was added as a carrier to a final concentration of 0.25 μg per ml. Trichloroacetic acid (TCA) (Mallinockrodt, Paris, KY) was added to a final concentration of 5% in each assay to
precipitate the undigested DNA substrate. Each reaction tube was kept in ice for 20 min. Following centrifugation at 3,000 rpm for 10 min (SAVANT, model PH40-11, Hicksville, NY), 200 µl of the acid-soluble nucleotide was removed from the supernatant for scintillation counting in a Beckman LS-7800 liquid scintillation counter. *E. coli* DNase I was used as a positive control in this assay.

**Purification of the chemically induced EBV-DNase.** The procedures of Goodman et al., 1978 (14) for harvesting the cells, making the crude cell extracts, and performing DEAE-cellulose, phosphocellulose, and DNA-cellulose column chromatography were followed with minor modifications: The cell extracts from 10 liters of Raji cells were centrifuged at 12,000 x g for 20 min in a SS-34 rotor in a Sorvall RC-2B centrifuge, and the resulting supernatant was loaded onto a DEAE-I-cellulose column (2 x 5 cm) that was equilibrated and eluted with 300 mM potassium phosphate buffer (pH 8.3) containing 20% glycerol (v/v), 1 mM phenylmethanesulfonylfluoride (PMSF) (Sigma, St. Louis, MO), 1 mM EDTA, and 2 mM dithiothreitol (DTT) (Boehringer Mannheim, Kuhl Lagern, W. Germany). The eluted samples in the peak fractions were pooled, dialysed against 20 mM potassium phosphate buffer (pH 8.3) at 4°C, and centrifuged at 12,000 x g for 20 min (Sorvall, SS-34 rotor). The dialysate was loaded onto another DEAE-II-cellulose column (2 x 5 cm). The EBV-DNase was eluted with a linear gradient of 50-300 mM potassium phosphate buffer (pH 8.3). Fractions of 3 ml from the DEAE-II-cellulose column were collected and assayed. Fractions containing DNase activity were pooled and dialysed against 1 liter of 20 mM potassium phosphate buffer (pH 8.3) for 15 hrs at 4°C. Dialysed DEAE-II-cellulose DNase peak fractions were loaded onto a phosphocellulose column (1.5 x 5 cm). A gradient elution from 50 to 300 mM potassium phosphate buffer (pH 8.3) was used. Fractions of 2 ml were collected and the enzyme activity was determined. The fractions
containing the EBV-DNase were pooled and dialysed against 1 liter of 20 mM potassium phosphate buffer (pH 8.3) for 15 hrs at 4°C.

Dialysed phosphocellulose DNase peak fractions were loaded onto a DNA-cellulose column (1.2 x 1 cm). A gradient elution of 50 to 400 mM potassium phosphate buffer (pH 8.3) was used. Fractions of 1 ml were collected and the enzyme activity was determined. The fractions containing the EBV-DNase were pooled and dialysed against 1 liter of dialysis buffer (20mM Tris-buffer (pH 8.3), 40% glycerol (v/v), 2 mM DTT, 2 mM PMSF) at 4°C overnight. The dialysed EBV-DNase was stored at -70°C until used.

**EBV-DNase neutralization assay.** Neutralization of EBV-DNase activity by human NPC sera (provided by Dr. J. F. Chiou, Taipei, and Dr. R. S. Tan, Beijing) and rabbit polyclonal was assayed as described by Cheng et al. (5). After incubation at room temperature for 20 min, the immunocomplexes were pelleted by centrifugation at 3,000 rpm for 10 min in a swinging bucket rotor (SAVANT, model PH40-11, Hicksville, NY), and the supernatant was examined for DNase enzyme activity (described above).

**SDS-PAGE, blotting, and immunostaining.** Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (23) using 10% gels. After electrophoresis, gels were silver stained by the method of Merril et al. (29). Proteins were transferred to nitrocellulose paper by the method of Bowen et al. (3). Immunostaining of proteins on nitrocellulose paper was carried out according to Legocki and Verma (24) by using alkaline phosphatase-conjugated goat antibodies (2).
**EBV-DNase determination by agarose gel electrophoretic analysis.**

Specific EBV-DNase nucleolytic reactions were carried out as described in the EBV-DNase assay. The reaction solution contained 50 μl of 50 mM Tris (pH 8.3), 10 mM MgCl₂, 10 mM β-mercaptoethanol, 2 μg of the BstE II-digested Lambda-DNA (Biolabs, Beverly, MA), and 4 μg of the partially purified EBV-DNase. The mixture was incubated in the presence and absence of NPC patient sera in a 37°C water bath with constant shaking. Five microliters (μl) of the reaction mixture containing 0.2 μg of BstE II-digested Lambda-DNA was removed at different time intervals and was put into a pre-cooled microcentrifuge tube for agarose gel electrophoretic analysis. The BstE II-digested Lambda-DNA was analysed in 1% horizontal agarose gel in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, 0.5 μg of ethidium bromide per ml, pH 8.0) (28). *E. coli* DNase I was used as a positive control, and BstE II-digested Lambda-DNA in the absence of enzyme preparation was used as a negative control.

**Gel-retardation analysis of the DNA-cellulose peak fraction B.** The DNA-binding affinity of the partially purified EBV-DNase was determined in polyacrylamide gel electrophoresis (PAGE). The DNA-cellulose peak fraction was used as enzyme to digest Hind III-digested Lambda-DNA (Biolabs, Beverly, MA). DNA binding was performed at 4°C in the absence of Mg²⁺, which is the required cation in this nucleolytic reaction. The reaction solution contained 50 μl of 50 mM Tris (pH 8.3), 10 mM β-mercaptoethanol, 0.2 μg of the Hind III-digested Lambda-DNA, and 8 μg of the partially purified EBV-DNase. The mixture was analysed by the PAGE at 4°C followed by 40 min of nucleolytic reaction. The same amount of the EBV-DNase in the absence of Hind III-digested Lambda-DNA was used as negative control.

**Radioiodination of the DNA-cellulose peak fraction B.** The chloramine-T procedure was used for radioiodination of proteins (19). The iodination
was carried out at room temperature. DNA-cellulose samples were incubated with 0.5 mCi of Na$^{125}$I (ICN, Costa Mesa, CA) and 160 µg of chloramine-T (Sigma, St. Louis, MO) in 400 µl of 50 mM potassium phosphate buffer, pH 8.3. Glycine was added to a final concentration of 15 µg/ml after a 1 min of iodination period to prevent any subsequent iodination. Separation of labelled EBV-DNase from unincorporated label was carried out by gel filtration using a column containing 30 mg of 4B dextran (Bio-Rad, Richmond, CA). Precipitation of iodinated EBV-DNase was done by mixing the labelled EBV-DNase with TCA to a final concentration of 10% at 4°C overnight.

**Production of rabbit polyclonal antibodies.** The partially purified EBV-DNase from the DNA-cellulose peak fractions (300 µg) was used for immunization to produce rabbit antiserum to the EBV-DNase. The rabbit was immunized by repeated intradermal injection. The first inoculum consisted of 0.25 ml of complete Freund adjuvant mixed with 0.25 ml of the antigen preparation (100 µg). The animal was boosted with incomplete Freund adjuvant once a month and bled from the ear 1 week after each booster. Pre-immuned serum was collected from the rabbit 1 week before immunization began. Sera from the injected rabbit were tested by dot immunostaining with partially purified EBV-DNase as antigen and screened for neutralization antibodies to the viral DNase by a neutralization assay.

**Radioimmunoprecipitation (RIP).** The iodinated DNA-cellulose peak fraction B was pre-absorbed by the EBV-negative human serum (provided by NIH) or pre-immuned rabbit serum before immunoprecipitation. The pre-absorbed supernatants were assayed for nuclease activity. Immunoprecipitations were carried out for 18 hrs at 4°C by using rabbit anti-DNase sera and human NPC sera. The resulting complexes were precipitated by the addition of protein A-sepharose (Sigma,
St. Louis, MO), followed by incubation at 4°C for 30 min with constant shaking. Protein A-sepharose and adhering immune complexes were collected by centrifugation and then washed 6 times in phosphate buffer saline (PBS), pH 7.3. Protein A-sepharose was separated from immune complex by boiling for 10 min in SDS-PAGE sample buffer (50 mM Tris, pH 7.3, 1% SDS (w/v), 20% glycerol (v/v), 5% β-mercaptoethanol (v/v), and 100 µg/ml of Bromphenol Blue). The protein A-sepharose was then pelleted, and the supernatant was removed and analysed by SDS-PAGE and autoradiography. The dried gel was exposed to Kodak X-AR film at -20°C.

**FPLC purification of DNA-cellulose peak fraction B.** Partially purified EBV-DNase from the peak fraction of the DNA-cellulose column was further fractionated by a Mono Q (HR 5/5) column using a Pharmacia high-resolution FPLC system (21). The Mono Q column was equilibrated with 10 mM sodium acetate buffer, pH 6.0, and a total of 40 µg of protein was loaded onto the column. The column was washed with 10 mM sodium acetate buffer, pH 6.0, and proteins were eluted with a linear gradient containing 10-1000 mM sodium acetate buffer, pH 6.0. The flow rate was 1 ml/min, and 1 ml fractions were collected. The nuclease activity of each fraction was determined and neutralization by human NPC sera was also assayed. The peak fractions with nuclease activity and neutralization activity were pooled, concentrated by TCA precipitation for three days, and then analysed by SDS-PAGE.
RESULTS

Purification of the chemically induced EBV-DNase in P3HR-1 and Raji cells. The combination of diterpene ester promoter of mouse skin tumors, TPA, and cell differentiation inducer, SB, was used to induce DNase activity in the Epstein-Barr virus-producer cell line P3HR-1 (37) and the Burkitt's tumor nonproducer Raji cell line (32). The chemically induced EBV-DNase from Raji cell lysates was partially purified by sequential ion-exchange column chromatography, and the profiles of each purification step are shown in Figs. 1, 2, and 3. Each peak fraction could be neutralized by human NPC sera. The chemically induced EBV-DNase in Raji cell extracts eluted from DEAE-II-cellulose at 125 mM (Fig. 1), phosphocellulose at 225 mM (Fig. 2), and DNA-cellulose at 250 mM potassium phosphate buffer (Fig. 3). Based upon these elution profiles and neutralization by human NPC sera, the chemically induced EBV-DNase is different from the cellular DNase (8, 35, 7).

In Tables 3 and 4 are summarized the total enzymatic activity, specific activity, yield, and purification in each of the column chromatographic steps. The specific enzyme activity of the partially purified DNase increased 70-fold over that of the crude extracts and represents a 5.4% recovery in induced-Raji cells (Table 3) and a 2.1% recovery from induced-P3HR-1 cells (Table 4).

The SDS-PAGE analysis of samples from different steps of EBV-DNase purification in Raji and P3HR-1 cell lines was visualized by silver staining. The results are shown in Figs. 4 and 5. The DNA-cellulose samples purified from Raji cells contained five major bands with the M.W. of 50 kilodaltons (KDa), 52 KDa, 58 KDa, 66 KDa, and 80 KDa (Fig. 4). However, the DNA-cellulose samples purified from P3HR-1 contained only two major proteins of 52 KDa and 58 KDa (Fig. 5).
Fig. 1. DEAE-II-cellulose column chromatography purification profile of EBV-DNase from TPA/SB-induced Raji cell extracts. The DNase activity was measured by the release of acid-soluble nucleotide from *E. coli* [3H]-DNA.
Fig. 2. Phosphocellulose column chromatography purification profile of EBV-DNase. Fractions 4 through 6 from DEAE-II-cellulose were pooled, dialysed, and then chromatographed as described under Materials and Methods. The DNase activity was measured by the release of acid-soluble nucleotide from *E. coli* [³H]-DNA.
Fractions 10 through 12 from phosphocellulose were pooled, dialysed, and then chromatographed as described under Materials and Methods. Peak fractions A and C cannot be inhibited by human NPC sera. Peak fractions B can be inhibited by human NPC sera. Fractions B was used in all subsequent experiments. The DNase activity was measured by the release of acid-soluble nucleotide from *E. coli*[^3H]-DNA.

Fig. 3. DNA cellulose column chromatography purification profile of EBV-DNase.
Table 3. Purification of EBV-DNase from TPA/SB-induced Raji cells

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (unit)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
<th>Purification</th>
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<tr>
<td>Crude extract</td>
<td>40.0</td>
<td>538.0</td>
<td>259.8</td>
<td>0.48</td>
<td>100.0</td>
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<tr>
<td>DEAE-II</td>
<td>24.0</td>
<td>34.5</td>
<td>240.0</td>
<td>2.94</td>
<td>92.3</td>
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<tr>
<td>Phosphocellulose</td>
<td>7.2</td>
<td>3.6</td>
<td>65.6</td>
<td>18.20</td>
<td>25.2</td>
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<tr>
<td>DNA-cellulose</td>
<td>1.0</td>
<td>0.4</td>
<td>14.1</td>
<td>35.25</td>
<td>5.4</td>
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a 10 liter of Raji cells were induced by 40 ng/ml of TPA and 4 mM of SB for three days.
b One unit of DNase activity was defined as that amount of activity which digests 1 µg of native DNA to acid-soluble nucleotide in 10 min at 37°C.

Table 4. Purification of EBV-DNase from TPA/SB-induced P3HR-1 cells

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (unit)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
<th>Purification</th>
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<tr>
<td>Crude extract</td>
<td>30.0</td>
<td>522.00</td>
<td>141.10</td>
<td>0.27</td>
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<td>DEAE-II</td>
<td>23.0</td>
<td>28.75</td>
<td>121.90</td>
<td>4.24</td>
<td>86.3</td>
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<tr>
<td>Phosphocellulose</td>
<td>10.0</td>
<td>5.10</td>
<td>75.60</td>
<td>14.28</td>
<td>53.5</td>
</tr>
<tr>
<td>DNA-cellulose</td>
<td>0.5</td>
<td>0.17</td>
<td>2.99</td>
<td>17.60</td>
<td>2.1</td>
</tr>
</tbody>
</table>

a 10 liter of P3HR-1 cells were induced by 40 ng/ml of TPA and 4 mM of SB for two and half days.
b One unit of DNase activity was defined as that amount of activity which digests 1 µg of native DNA to acid-soluble nucleotide in 10 min at 37°C.
Fig. 4. SDS-PAGE of samples from the peak fractions of the three columns used in the purification of EBV-DNase from the Raji cell extracts. Lanes A through E are described as follows: DNA-cellulose (Lane A), phosphocellulose (lane B), DEAE-II-cellulose (lane C), cell extracts (lane D), and molecular weight standards (lane E). Lanes A through E were visualized by silver staining. Proteins from these peak fractions and cell extracts were used in immunoblots. Proteins from DNA-cellulose peak fraction B were used in all subsequent radiiodination and FPLC.
Fig. 5. SDS-PAGE of samples from the peak fractions of the three columns used in the purification of EBV-DNase from the P3HR-1 cell extracts. Lanes A through F are described as follows: molecular weight standard (lane A), cell extracts (lane B), DEAE-II-cellulose (lane C), phosphocellulose (lane D), and DNA-cellulose (lanes E and F). Lanes A through F were visualized by silver staining.
Characterization of the chemically induced EBV-DNase. It has been observed that human NPC sera neutralized up to 86% of the EBV-DNase activity in partially purified samples (5). The presence of EBV-DNase was verified by the results shown in Table 5. The DNase activity was neutralized up to 90% by human NPC sera and 54% by rabbit polyclonal antibodies when compared with normal human sera and pre-immuned serum.

Considering the potential DNA affinity of EBV-DNase, a gel-retardation experiment was carried out in PAGE. The DNA-cellulose peak fraction B (Fig. 3) was mixed with Hind III-digested Lambda-DNA in test tubes at 4°C in the absence of MgCl₂, the required divalent cation in this nucleolytic reaction (8). The large fragments of Hind III-digested Lambda-DNA were retarded at the stacking gel by binding with the EBV-DNase in the DNA-cellulose samples. The results are shown in Fig. 6. After silver staining, proteins could not be visualized in the lane containing both Lambda-DNA and partially purified EBV-DNase; however, proteins could be seen in the lane containing only partially purified EBV-DNase. These results indicate that the partially purified EBV-DNase has a DNA-binding affinity which causes non-visualized proteins at the running gel of PAGE. Moreover, in the absence of Mg²⁺, the nucleolytic reaction can not occur.

The partially purified EBV-DNase digested both native and denatured DNA to mononucleotides (8). To determine whether restriction-enzyme-digested Lambda-DNA could be used as substrate, and if the reaction could be visualized by staining instead of the standard isotopic assay, the nuclease activity of EBV-DNase on BstE II-digested Lambda-DNA and its neutralization by human NPC sera were investigated in agarose gel electrophoresis. The DNA-cellulose peak fraction B (Fig. 3) was used as enzyme to digest BstE II-digested Lambda-DNA. The results are shown in Fig. 7. In lanes 2 to 5 of Fig. 7 it is evident that partially purified EBV-DNase digested Lambda-DNA in 1% agarose gel in the different intervals. In lanes 6 to 14 it is evident that the nuclease
activity of the partially purified EBV-DNase could be neutralized by the addition of human NPC sera prior to the commencement of the 40 min nucleolytic reaction. These results indicate that the partially purified EBV-DNase could digest Lambda-DNA. Human NPC sera could neutralize the nuclease activity completely. DNA digestion required Mg$^{2+}$ and the nonisotopic assay could be easily visualized.

Table 5. Inhibition$^a$ of EBV-DNase by 1:20 dilution of human NPC sera and rabbit polyclonal antisera

<table>
<thead>
<tr>
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<th>Non-induced cell lysate</th>
<th>Crude extracts</th>
<th>Purified EBV-DNase</th>
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</thead>
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<tr>
<td>Enzyme activity$^b$ (no serum)</td>
<td>340$^c$</td>
<td>1807</td>
<td>4196</td>
</tr>
<tr>
<td>Normal sera</td>
<td>349 (N)$^d$</td>
<td>2010 (N)</td>
<td>3580±267 (14)</td>
</tr>
<tr>
<td>Human NPC sera</td>
<td>204 (40)</td>
<td>408 (77)</td>
<td>422±23 (90)</td>
</tr>
<tr>
<td>Enzyme activity (no serum)</td>
<td>340</td>
<td>1583</td>
<td>3204</td>
</tr>
<tr>
<td>Pre-immuned sera</td>
<td>253 (0)</td>
<td>1721 (N)</td>
<td>3090±31 (4)</td>
</tr>
<tr>
<td>Rabbit antisera</td>
<td>234 (31)</td>
<td>641 (60)</td>
<td>1483±196 (54)</td>
</tr>
</tbody>
</table>

$^a$ Inhibition was defined as that amount of decreased activity of EBV-DNase in the presence of each corresponding antibody.

$^b$ Nuclease activity assays were performed as described under Materials and Methods.

$^c$ Radioactivity in cpm as determined by scintillation counting.

$^d$ Values in parentheses represent % of inhibition, and N in parentheses represent no inhibition.
Fig. 6. Determination of the DNA-binding affinity and the Mg$^{2+}$ requirement of the partially purified EBV-DNase in a 12.5% PAGE. Molecular weight standards (lane A), DNA-cellulose peak fraction B mixed with Hind III-digested Lambda-DNA in the absence of the divalent cation (Mg$^{2+}$) at 4°C (lane B), DNA-cellulose peak fraction B (lane C), and Hind III-digested Lambda-DNA (lane D).
Fig. 7. Determination of the partially purified EBV-DNase activity in a 1% agarose gel. Each lane contains 0.2 μg of BstE II-digested Lambda-DNA. Lanes 1 through 15 are described as follows:

BstE II-digested Lambda-DNA which has 14 fragments with different sizes (lane 1), BstE II-digested Lambda-DNA digested by EBV-DNase for 20, 40, 60, and 80 min (lanes 2 to 5, respectively), BstE II-digested Lambda-DNA digested by EBV-DNase for 20, 40, 60, and 80 min of a nucleolytic reaction to which a 1/20 dilution of human NPC sera had been added (lanes 6 to 9, respectively), different dilutions (1/10, 1/20, 1/40, 1/60, 1/80) of human NPC sera were added prior to a 40 min nucleolytic reaction (lanes 10 to 14, respectively), and BstE II-digested Lambda-DNA by E. coli DNase I (lane 15).
Determination of the M.W. of the chemically induced EBV-DNase. Due to the low concentration of protein in the column eluants, the volumes of the peak fractions were reduced prior to protein analysis by SDS-PAGE. Despite these efforts, the concentrated nuclease-active fraction from DNA-cellulose chromatography produced only a faint protein banding pattern in SDS-PAGE. The results are shown in Figs. 4 and 5. However, the M.W. of chemically induced EBV-DNase has been reported (20, 8, 32) with a M.W. ranging between 70 KDa and 58 KDa (see Table 1).

Three methods were used to determine the M.W. of the chemically induced EBV-DNase from TPA/SB-induced Raji cells. They were immunostaining, radioimmunoprecipitation (RIP), and the ion-exchange chromatography using FPLC. The immunostaining data are shown in the western blots using human NPC sera (Fig. 8) and rabbit polyclonal antibodies (Fig. 9). The results show a different reaction of human NPC sera and rabbit antisera to the antigen mixtures of different stages of purification. The human NPC sera showed broad reactions to the antigens in the range of 45 KDa and 66 KDa. The rabbit antiserum showed a strong reaction to 58 KDa and 52 KDa proteins, even in the DNA-cellulose sample.

The small amount of EBV-DNase could not be detected in western blots (Fig. 8, lane A). Radioimmunoprecipitation was applied for enhancing the sensitivity in detecting the EBV-DNase. Equal amounts of DNA-cellulose samples were iodinated and then immunoprecipitated with the human EBV-negative sera, human NPC sera, pre-immuned rabbit sera, and the rabbit polyclonal antisera. The resulting complexes were analysed by SDS-PAGE. An autoradiogram is shown in Fig. 10. To further reduce the non-specific background, iodinated samples were pre-absorbed with the human EBV-negative sera or pre-immuned rabbit sera, and the pre-absorbed supernatants were then immunoprecipitated by human NPC sera or rabbit polyclonal antiserum. The results are shown in Fig. 11.
The third approach to determine the M.W. of the EBV-associated DNase was to further purify this enzyme to homogeneity by FPLC. FPLC purification profile is shown in Fig. 14. Both FPLC peak fraction A and B had a nuclease activity, but only peak A was neutralizable by human NPC sera (Table 6). The specific activity of the peak-A fraction was 185 units/mg and a purification of 385-fold was achieved, but the yield was only 1.7% (Table 7). SDS-PAGE analysis of FPLC peak fraction A and B is shown in Fig.13. The results showed that a small amount of the chemically induced EBV-associated DNase that could be neutralized by human NPC sera was purified to the homogeneity, as determined by silver staining (Fig.13, lane A) and had a M.W. of 58 KDa. The two proteins with M.W. of 58 KDa and 52 KDa present in fraction B (Fig.13, lane D) also had nuclease activity that was not neutralized by human NPC sera (Table 6). These results indicate the potential presence of two different DNases in the TPA/SB-induced Raji cell extracts.
Fig. 8. Western immunoblot of polypeptides from the peak fractions of the three columns and TPA/SB-induced Raji cell extracts bound with human NPC sera at 1:100 dilution in TBST buffer. DNA-cellulose peak fraction B (lane A), phosphocellulose (lane B), DEAE-II-cellulose (lane C), and cell extracts (lane D) were immunostained by alkaline phosphatase-conjugated goat anti-human IgG. Molecular weight standards (lane E) and 58 KDa pre-stained protein marker (pyruvate kinase from chicken muscle) (lane F) were stained by Amido Black staining.
Fig. 9. Western immunoblot of polypeptides from the peak fractions of the three columns and TPA/SB-induced Raji cell extracts bound with rabbit polyclonal antisera at 1:100 dilution in TBST buffer. Cell extracts (lane A), DEAE-II-cellulose (lane B), phosphocellulose (lane C), and DNA-cellulose peak fraction B (lane D) were immunostained by alkaline phosphatase-conjugated goat anti-rabbit IgG. 58 KDa pre-stained protein marker (lane E) and molecular weight standards (lane F) were stained by Amido Black.
Fig. 10. Autoradiogram of radioimmunoprecipitations of the iodinated EBV-DNase from the DNA-cellulose peak fraction by 1:20 dilution of each of the corresponding antibodies: EBV-negative human sera (lane A), human NPC sera (lane B), pre-immunized rabbit sera (lane C), and rabbit polyclonal antisera (lane D). The adhering immune complexes were pelleted by protein A-sepharose and analysed by 10% SDS-PAGE as described under Materials and Methods.
Fig. 11. Autoradiogram of radioimmunoprecipitations of the iodinated EBV-DNase from the DNA-cellulose peak fraction B by 1:20 dilution of human NPC sera (lane A) and rabbit polyclonal antisera (lane B). The iodinated DNA-cellulose peak fraction B was pre-absorbed by the human EBV-negative sera or pre-immuned rabbit sera. The adhering immune complexes were pelleted by protein A-sepharose and analysed by 10% SDS-PAGE as described under Materials and Methods.
Fig. 12. FPLC Mono Q column chromatography of the DNA-cellulose peak fraction B. The DNA-cellulose peak fraction B containing high nuclease activity and neutralizable activity by human NPC sera were run through a Mono Q column equilibrated in 10 mM sodium acetate buffer (pH 6.0) and eluted with a 10 to 1000 mM sodium acetate linear gradient. Pooled peak fractions were precipitated in 15% TCA for 3 days at 4°C and then analysed by SDS-PAGE.
Table 6. Inhibition\(^a\) of EBV-DNase from FPLC fractions by 1:20 dilution of human NPC sera

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>9</th>
<th>10</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase activity(^b)</td>
<td>817(\pm)81</td>
<td>507(\pm)334</td>
<td>4158(\pm)282</td>
<td>1498(\pm)73</td>
</tr>
<tr>
<td>NPC sera added</td>
<td>517</td>
<td>429</td>
<td>621(\pm)295 (85)(^d)</td>
<td>2437(\pm)154 (N)</td>
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</table>

\(^a\) Inhibition is defined as that amount of decreased activity of EBV-DNase in the presence of 1:20 dilution of human NPC sera.

\(^b\) Nuclease activity assays were performed as described under Materials and Methods.

\(^c\) Radioactivity in cpm as determined by scintillation counting.

\(^d\) Values in parenthesis represent % of inhibition, and N in parenthesis represent no inhibition.

Table 7. Purification of EBV-DNase from DNA-cellulose peak B fraction by FPLC

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (unit)(^a)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
<th>Purification (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>40.0</td>
<td>538.0</td>
<td>259.8</td>
<td>0.48</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>DNA-cellulose</td>
<td>1.0</td>
<td>0.4</td>
<td>14.1</td>
<td>35.25</td>
<td>5.4</td>
<td>73.4</td>
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<td>FPLC(^b)</td>
<td>2.0</td>
<td>0.0244</td>
<td>4.5</td>
<td>185.25</td>
<td>1.7</td>
<td>385.9</td>
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\(^a\) One unit of DNase activity is defined as that amount of activity which digests 1 \(\mu\)g of native DNA to acid-soluble nucleotide in 10 min at 37°C.

\(^b\) Peak fraction B containing 40 \(\mu\)g of partially purified EBV-DNase was loaded onto a Mono Q (HR 5/5) column.
Fig. 13. SDS-PAGE of samples from FPLC peak fractions. Lanes A through D were visualized by silver staining.
Lane A: peak A fraction of Mono Q column.
Lane B: Molecular weight standards.
Lane C: 58 KDa protein marker.
Lane D: peak B fraction of Mono Q column.
DISCUSSION

The data presented in this thesis show that an EBV-DNase can be chemically induced by TPASB in Raji and P3HR-1 cell lines. Induced cells exhibit increased expression of the EBV-associated DNase as compared with the non-induced Raji cells and P3HR-1 cells. When the chemically induced Raji and P3HR-1 cells express EBV-DNase activity, this is an indication that the cells are undergoing active EBV DNA replication after latency activation. Ooka et al. demonstrated that the chemically induced DNase activity in Raji cells can be neutralized by the EBV-positive sera, mainly by sera from NPC patients (32). The chemically induced EBV-DNase activity was shown to be 90% neutralizable with human NPC sera but not with EBV-negative human sera. Furthermore, the rabbit antisera can also partially inhibit 54% of the EBV-DNase activity with the same property as human NPC serum does (Table 5).

The partial purification of the chemically induced EBV-associated DNase from the cell extracts was accomplished by sequential ion-exchange column chromatography. The viral nuclease activity from Raji cell extracts eluted from DEAE-II-cellulose at low salt concentration, and from phosphocellulose and DNA-cellulose at high salt concentration (Figs. 1, 2, 3). These results are similar to those reported by Clough (9). After DEAE-cellulose, phosphocellulose, and finally DNA-cellulose chromatography, the nuclease showed a net purification of about 70-fold in both cell lines. The yield was about 5.4% of the total EBV-induced enzyme in the Raji crude extracts but only 2.1% from P3HR-1 cell lysate (Tables 3, 4).

The chemically induced EBV-DNase, like those induced by herpes simplex virus types 1 and 2, has characteristics of an optimum alkaline pH, a strong requirement for Mg$^{2+}$, and is unstable when highly purified (18). Clough demonstrated that the EBV-DNase isolated from P3HR-1 cells utilizes both single- and double-stranded DNA as template, reducing both to monophosphonucleotides (8). All of these characteristics
were confirmed by the investigation of the partially purified EBV-DNase from both TPA/SB-induced Raji and P3HR-1 cells.

The partially purified EBV-DNase exhibits a DNA-binding affinity as shown in a gel retardation analysis (Fig. 6). These data also confirm that the requirement of the divalent cation (Mg$^{2+}$) is important in its nucleolytic reaction. Furthermore, the partially purified EBV-DNase can use the BstE II-digested Lamda-DNA as substrate for digestion, and the nuclease activity can be completely neutralized by human NPC sera (Fig. 7). The kinetics of digestion by EBV-DNase can be visualized by simple staining, and this nonisotopic assay provides an alternative method for the standard isotopic assay for EBV-DNase determination.

The partially purified EBV-DNase is different from the cellular DNase based upon elution profile, optimum alkaline pH (pH 8.3), divalent cation requirement, substrate specificity, and neutralization by sera from NPC patients (8, 35, 7). Due to the limited recovery and stability of partially purified sample, the M.W. of EBV-DNase has not been determined accurately. However, the partially purified EBV-DNase has been reported to have a sedimentation coefficient of about 4S (Svedberg unit) (9, 32). A globular protein of this sedimentation coefficient value would have a M.W. of approximately 58 KDa (27). SDS-PAGE analysis of EBV-DNase purified from Raji and P3HR-1 cell lines showed two similar polypeptides, 58 KDa and 52 KDa in the DNA-cellulose peak fraction (Figs. 4, 5). The M.W. of 58 KDa supports the assignment of the size of EBV-DNase with 4S. The 52 KDa protein can be either a second EBV-DNase or a host DNase contaminant.

Studies from immunostaining of western blots show that (i) the anti-EBV DNase rabbit antisera strongly reacted with the 52 KDa and 58 KDa proteins of the DNA-cellulose samples (Fig. 9), and (ii) the human NPC sera reacted with several proteins including the 58 KDa and 52 KDa proteins (Fig. 8). This information indicates that 58 KDa and 52 KDa could be the M.W. of the chemically induced EBV-DNase.
Studies from radioimmunoprecipitation using rabbit anti-DNase sera, pre-immuned rabbit sera, the EBV-negative, and EBV-positive human sera show that the rabbit antisera and human NPC sera precipitated a 58 KDa protein (Figs. 10, 11). There was no indication for specific precipitation of a protein with similar M.W. by the rabbit pre-immuned serum and human EBV-negative serum. These studies indicate that the chemically induced EBV-DNase has a M.W. of 58 KDa.

EBV-DNase was also purified to apparent homogeneity by FPLC (Fig.13), and a 385-fold purification was achieved. The specific activity of the highly purified enzyme is 185 units/mg. However, the final yield of this enzyme was only about 1.7% of the total EBV-DNase in the Raji cell extracts (Table 7). Two nuclease activity peaks were identified in FPLC (Fig.12). Peak A had nuclease activity and was neutralizable by NPC patient sera. SDS-PAGE analysis of peak A had a single protein band with a M.W. of 58 KDa (Table 6). Peak B appeared to contain two proteins, 58 KDa and 52 KDa, but the associated nuclease activity was not neutralizable by human NPC sera (Fig.13), suggesting the presence of a second EBV-DNase or representation of a co-purified host DNase contaminant. These studies strongly suggest that the chemically induced EBV-DNase has a M.W. of 58 KDa.

The chemically induced Raji and P3HR-1 cells exhibit increased EBV-DNase nuclease activity, but non-induced Raji and P3HR-1 cells do not show this increase. This partially purified EBV-DNase is neutralized by human NPC sera and rabbit polyclonal antibodies but can not be neutralized by EBV-negative human sera or pre-immuned rabbit sera. The chemically induced EBV-DNase purified by sequential ion-exchange column chromatography has the following characteristics: an optimum alkaline pH (pH 8.3), a high salt resistance in EBV-DNase activity assay, and ability to use Lambda-DNA as substrate. By using three different approaches, which include immunostaining, radioimmunoprecipitation and FPLC, the M.W. of the chemically induced EBV-DNase has been determined to be 58 KDa. This 58 KDa protein that was
purified to homogeneity shown as a single protein species in SDS-PAGE analysis has the highest EBV-DNase activity and is inhibited by both polyclonal rabbit antisera and human NPC sera. In previous reports, the determination of the M.W. of EBV-DNase was based on enzyme samples which had not been purified to homogeneity (20, 8, 35, 32).
REFERENCES


CURRICULUM VITA

NAME: GUANG-YUH HWANG

SOCIAL SECURITY NUMBER: 528 59 2776

BIRTHDATE: 8 OCTOBER 1960

EDUCATION:

<table>
<thead>
<tr>
<th>Institution</th>
<th>Degree</th>
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<td>M.S.</td>
<td>1989</td>
<td>Molecular Biology</td>
</tr>
<tr>
<td>Tunghai University, Taiwan</td>
<td>B.S.</td>
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<td>Biology</td>
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RESEARCH EXPERIENCE:


1986-1987: Purification of a chemically induced EBV-DNase from Raji and P3HR-1 cells.


PROFESSIONAL SOCIETY:

American Microbiology Society

Society of Chinese Bioscientists in America

PUBLICATION: