2m11.2715.5

Université de Montréal

Isolation of Xeroderma Pigmentosum-Variant Complementing Factor from Human 293 Cells

par

Chunli Lei

Département de Sciences Biomédicales

Faculté de Médecine

Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de
Maître ès sciences (M.Sc.)
en sciences biomédicales

Octobre 1998

© Chunli Lei, 1998

W
4
U58
1999
v.112

Université de Montréal
Faculté des études supérieures

Ce mémoire intitulé:

Isolation of Xeroderma Pigmentosum-Variant Complementing Factor
from Human 293 Cells

présenté par:

Chunli Lei

a été évalué par un jury composé des personnes suivantes:

Président rapporteur:	Dr. Elliot Drobetsky
Directeur de recherche:	Dr. Daniel Lee Svoboda
Membre du jury:	Dr. Alain Bonnardeaux

Mémoire accepté le: 99.04.01

Résumé

La maladie Xéroderma Pigmentosum (XP) est une maladie récessive autosomale présentant une incidence élevée de cancer de la peau. Parmi les différents sous-groupes (ou sous-unités) de XP, seul le groupe XP variant (XP-V) présente un taux plus lent de synthèse d'ADN après une exposition aux rayons UV. Ces cellules, comparées aux cellules normales, sont légèrement plus sensibles à l'effet cytotoxique des radiations UV, puisque XP-V est caractérisé par un défaut de synthèse translésionnelle (translesion synthesis). Dans un essai *in vitro* de réplication d'ADN, certains facteurs restaurent la faculté des extraits cellulaires XP-V de répliquer une matrice (template) d'ADN contenant un dimère de thymine (T<>T). Ces facteurs correctifs peuvent être isolés et déterminés.

Pour isoler ces facteurs correctifs utilisés par XP-V, de grande quantité de cellules rénales embryonnaires humaines (les cellules 293) ont été cultivées en suspension, en phase semi-log jusqu'à une densité de 0.5-0.6 x 10^6 cellules/ml. Les cellules resuspendues dans un milieu hypotonique ont été endommagées à l'aide d'un homogénéisateur Dounce; les noyaux ont été éliminés après ultracentrifugation . La concentration finale de protéines dans les extraits cytoplasmiques de cellules 293 était de l'ordre de 5.0-10.4 mg/ml. La préparation de la matrice d'ADN avec un dimère de thymine (T<>T) et celui du brin contrôle non endommagé (TT), ont été produits en hybridant un oligomère de 20 nucléotides contenant les bases non endommagées (TT) ou endommagées (T<>T) à un ADN circulaire simple brin (ssADN). L'ADN simple brin a été marqué avec du ^{3}H-TTP pour obtenir un ADN marqué au ^{3}H comme standard interne (^{3}H-ADN).

La réplication in vitro de la matrice d'ADN double brin contenant une origine SV-40 et un seul T<>T (ou TT) se fait en présence d'extraits de cellules normales (293) ou de cellules XP-V (CTAG) avec de l'antigène grand T et des dNTPs. La

réaction est arretée par l'addition de SDS et de protéinase K. Après l'incorporation des éléments radioactifs ^{32}P et ^{3}H, l'ADN produit est séparé sur une colonne de gel de filtration et analysé par électrophorèse sur un gel d'agarose 0.8% après avoir été digéré avec MfeI/MboI/DpnI. L'activité de réplication a été quantifiée en utilisant le logiciel du phosphorimager. Seuls les extraits cellulaires qui avaient une activité de synthèse translésionnelle (bypass) ont été utilisés pour être purifiés par chromatographie.

Le fractionnement des extraits cellulaires 293 par chromatographie échangeuse de cation en utilisant une colonne de phosphocellulose (P11) a donné deux pics de protéines: la fraction CFI et la fraction CFII. La fraction CFII avait une activité de synthèse translésionnelle, et la quantification par phosphorimager des unités récupérées était de l'ordre de 33.6-45.2%. Ceci confirme que le facteur de complémentation peut être partiellement purifié sur la colonne P11. Afin de purifier davantage le complexe protéique, la fraction CFII a été passée sur un tamis moléculaire, la résine Séphacryl 200 High Resolution. Plusieurs fractions correspondant a un seul pic de protéines non différentiées ont été obtenues et ces fractions avaient une activié de synthèse translésionnelle. Par la suite ce pool de fractions a été passé sur une colonne de type hydrophobique, une colonne Phényl sépharose. Nous n'avons pu détecté aucune activité de synthèse translésionnelle dans les fractions obtenues, même en les regroupant. La fraction CFII a été passée sur une colonne Q Sépharose-FF et éluée par gradient de densité. Nous avons obtenu cinq fractions. Seulement une ou deux fractions semblaient avoir une faible activité de synthèse translésionnelle.

Basé sur ces résultats nous avons démontré que le fractionnement des extraits cytoplasmiques des cellules 293 par des méthodes usuelles de purification des protéines a conduit à une purification partielle du facteur de complémentation. L'efficacité et la reproductibilité de l'essai de réplication *in vitro* d'ADN nous a

permis de démontrer que l'activité de synthèse translésionnelle dans les extraits de cellules C-TAG peut être restauré en utilisant des fractions recueillies d'extraits de 293 après séparation sur une colonne de phosphocellulose . Les résultats précédents confirment qu'il est possible de purifier le ou les facteur(s) de complémentation à partir de cellules humaines. Une purification plus poussée ouvrira la porte à l'étude des mécanismes biochimiques et moléculaires qui entrent en jeu dans la réplication d'ADN contenant un dimère de thymine. La séparation du facteur correctif impliqué dans XP-V nous aidera à isoler le gène XP-V, et à acquérir une meilleure compréhension de son rôle dans les transformations néoplasiques

Abstract

Xeroderma Pigmentosum (XP) is an autosomal recessive disorder with high incidence of skin cancer, but only the XP variant group (XP-V) has a slower rate of DNA synthesis after UV irradiation. They are only slightly more sensitive than normal cells to the cytotoxic effect of UV radiation, since XP-V cells carry a defect in replication of UV-induced DNA damage, defect in translesion synthesis. It is possible to isolate the correcting factor(s) which restore the ability of XP-V cell extracts to replicate a DNA template containing thymine dimer (T<>T) by *in vitro* DNA replication assay.

To isolate the Xeroderma pigmentosum-variant correcting factor, large quantity of human 293 cells were grown to mid-log phase of 0.5-0.6 x 10^6 cells/ml in spinner flask and disrupted by Dounce homogenizer in hypotonic buffer. The nuclei were removed by ultracentrifugation. The final concentration of 293 cell extracts was in the range of 5.0-10.24 mg/ml. The thymine dimer (T<>T) template and undamaged (TT) control template were prepared by annealing the original undamaged or T<>T containing 20 nucleotide oligomer to a circular, single-stranded DNA (ssDNA). The annealed ssDNA was labeled with [^{3}H]-TTP to get [^{3}H]-DNA internal standard.

The replication of SV40-based double-stranded DNA template carrying a single T<>T (or TT) was incubated with cell extracts from normal (293) or XP-V (CTAG) cells in the presence of T antigen and dNTPs at 37°C. The reaction was quenched by the addition of SDS and proteinase K. The production of DNA was isolated by gel filtration and analyzed by 0.8 % agarose gel electrophoresis after incorporation of the radioactivity [^{32}P] and [^{3}H], followed by the digestion with MfeI/MboI/DpnI. The replication activity was quantitated by phosphorimager. Only the cell extracts with bypass activity were used to run the columns.

Two peak fractions CFI and CFII were obtained by fractionation of the 293 cell extracts on a cation exchanger chromatography column-phosphocellulose (P-11). The

protein recovery of CFI, CFII was 52.09 %, 27.74 %, respectively. Only the high salt fraction CFII had bypass activity, the replication unit recovery quantitated by phosphorimager was 33.57-45.16 %. This confirms that complementing factor can be partially purified by P-11 column. The protein complex CFII was further fractionated by another 2 columns: size chromatography-Sephacryl high resolution 200 column, where the fractions through one protein peak had bypass activity and the hydrophobic chromatography-Phenyl sepharose column, where no bypass activity was detected even from the mixture of the fractions. CFII was loaded directly onto the Q sepharose-FF column by step gradient to get five fractions. Only one or two fractions appeared to have bypass activity, and the signals were not very strong.

These results showed that fractionation of 293 cell extracts yielded partially purified complementing factor by a standard protein purification procedure. The effective and reproducible *in vitro* DNA replication assay system using a fraction purified by Phosphocellulose chromatography, demonstrated 293 cells can complement XP-V defect. All these biochemical activities confirm that it is feasible to purify the complementing factor(s) from human cells. Further purification will open a door to study the biochemical mechanism of replicated DNA on damaged template. Isolation of the XP-V correcting factor will lead to isolation of the XP-V gene, and an understanding of its role in neoplasmic transformation.

TABLE OF CONTENT

TITLE PAGE....................i
IDENTIFICATION OF JURY....................ii
RÉSUMÉ....................iii-v
ABSTRACT....................vi-vii
TABLE OF CONTENTS....................viii-xii
LIST OF FIGURES....................xiii
LIST OF TABLES....................xiv
LIST OF ABBREVIATIONS....................xv-xvi
ACKNOWLEDGEMENTS....................xvii
DEDICATION....................xviii

I. INTRODUCTION....................1-25
I.1. DNA damage....................1-3
I.1.1. importance of DNA....................1
I.1.2. DNA damage....................1-3
I.1.2.1. types and consequences of DNA damage....................1-3
I.1.2.1.1. types of DNA damage....................1-2
I.1.2.1.2. consequences of DNA damage....................2-3
I.2. DNA repair....................3-6
I.2.1. pathways of DNA repair....................3-4
I.2.2. NER is the most frequent pathway for the removal of DNA damage...4-6
I.2.2.1. NER and UV radiation....................4-5
I.2.2.2. enzymatic mechanism of NER in *E. Coli*....................5-6
I.3. NER-defect diseases....................6-8
I.3.1. CS and TTD....................6-7
I.3.2. XP complementation groups....................7-8
I.4. XP-V....................8-11
I.4.1. experimental evidence of XP-V....................9

I.4.2. discovery of XP-V mutagenesis by shuttle vector....9-10

I.4.3. unclear mechanism of describing XP-V....10-11

I.5. relationship of XP-V and postreplication repair (PRR)....11-18

I.5.1. definition of PRR....11

I.5.2. possible mechanism of PRR....11-16

I.5.2.1. translesion synthesis....11-13

I.5.2.2. template switching....13-14

I.5.2.3. recombinational repair....15-16

I.5.3. the mechanism to elucidate XP-V defect in NER is still unclear....16-18

I.5.3.1. experimental evidence....17-18

I.5.3.2. requirement of the approaches to detect XP-V defect....18

I.6. *in vitro* DNA replication assay....18-24

I.6.1. SV40 DNA replication assay and the importance of T antigen....18-19

I.6.2. proteins of reconstitute system by *in vitro* assay....19-20

I.6.2.1. replication protein A....20

I.6.2.2. proliferating cell nuclear antigen....20-21

I.6.2.3. replication factor C....21

I.6.2.4. XPA....21-22

I.6.2.5. transcription factor....22

I.6.2.6. DNA polymerase....22-23

I.6.2.7. topoisomerase I....23

I.6.2.8. XPC-hHR23B....24

I.7. current development to detect XP-V defect by *in vitro* assay system....24

I.8. objective of present studies....24-25

II. MATERIAL AND METHODS....26-44

II.1. MATERIALS....26-32

II.1.1. Chemical Reagents/Enzymes/Culture Media and Plastic Culture flasks....26-29

II.1.1.1 Chemical Reagents/drugs....26-27

II.1.1.2. Enzymes....27-28
II.1.1.3. Cell lines/Cell Culture Media and Supplements/Culture flasks....28-29
II.1.1.3.1 Cell Lines....28
II.1.2.3.2. Culture Media and supplements....28-29
II.1.1.3.3. Culture Flasks....29
II.1.2. Stock Solutions/Buffers....29-31
II.1.2.1. Stock Solution....29
II.1.2.2. Buffers....29-30
II.1.2.2.1. NEB buffer #4....30
II.1.2.2.2. buffers for cell extracts....30
II.1.2.2.2.1. 1 x PBS....30
II.1.2.2.2.2. Hypotonic buffer....30
II.1.2.2.2.3. Isotonic buffer....30
II.1.2.2.3. buffer for Q-sepharose column....30
II.1.2.2.4. buffers for cellulose phosphate (P-11) column....30
II.1.2.2.4.1.buffer H + 15 mM or 100 mM KCl....30
II.1.2.3.4.2. buffer F +100mM KCl....30
II.1.2.3.4.3. buffer P + 500mM or 2M KCl....30
II.1.2.5. buffers for phenyl sepharose column....30
II.1.2.5.1. starting buffer....30
II.1.2.5.2. elution buffer....30
II.1.2.6. 1 x TBE buffer....30
II.1.2.7. 10 x annealing buffer....30
II.1.2.8. 10 x synthesis buffer for second strand synthesis....31
II.1.2.9. 20 x dNTP....31

II.1.2.10. 5 x T_7 sequenase reaction buffer............31
II.1.2.11. LB-agar medium..................................31
II.1.2.12.. 2 x YT medium..................................31
II.1.3. Isotopes..31
II.1.4. columns/resins...31-32
II.1.5. special equipment and products...32
II.2. METHODS..33-44
II.2.1. Cell cultures...33
II.2.2. Preparation of cell extracts...34-35
II.2.2.1. 293 cell extracts (293-S100).....................................34-35
II.2.2.2. CTAG cell extracts..35
II.2.3. Preparation of DNA templates...36-37
II.2.4. Internal standard preparation...37
II.2.5. In vitro DNA replication assay...37-41
II.2.6. Quantitation of bypass replication...41-42
II.2.7. Packing and running of phosphocellulose (P-11) column............42-43
II.2.8. running of Sephacryl high resolution (HR) 200 column.............43-44
II.2.9. Phenyl sepharose column...44
II.2.10 packing and running of Q-sepharose column................................44

III. RESULTS..45-55
III.1. bypass activity in 293 cell extracts...45-46
III.2. Scheme of fractionation of 293 cell-free extracts 293-S100 for the XP-V correcting factor purification procedure....................................47
III.3. high salt fraction of phosphocellulose column from 293 cell extracts (293-S100) can complement XP-V defect on thymine dimer template............47-51
III.4. Sephacryl HR-200 column fraction showed bypass activity,but did not separate well..51-53
III.5. Phenyl sepharose column cannot collect any bypass activity.................53-54
III.6. Q-Sepharose column fractions appear to have bypass activity................54-55

IV.DISCUSSION..56-65

IV.1. DNA repair and XP..56

IV.2. *in vitro* SV40 DNA replication assay and 293 cell extracts....................57-59

IV.3. High salt fraction of phosphate cellulose column had bypass activity.......59-61

IV.4. Sephacryl HR-200 column fractions had bypass activity, but didn't separate well..61-62

IV.5. Use of Phenyl sepharose column results in loss of bypass activity...........62-63

IV.6. Fractions of Q-Sepharose column appear to have bypass activity............63-65

V. CONCLUSION..66

VI. FUTURE WORKS..67

VII. REFERENCES..68-84

LIST OF FIGURES

Figure 1 Structure of cyclobutane pyrimidine dimer and pyrimidine-pyrimidone (6- 4) photoproduct by UV irradiation....2

Figure 2 Consequences of DNA damage.DNA damage cause mutagenesis, carcinogenesis,mutation and cell death....3

Figure 3 DNA repair pathways: base excision repair, nucleotide excision repair and mismatch ......repair....4

Figure 4 UV radiation spectrum....5

Figure 5 Model of postreplication repair—translesion synthesis....12

Figure 6 Models for replication on damaged templates....12

Figure 7 Model of postreplication repair—template switching....14

Figure 8 Model of postreplication repair—recombinational repair....15

Figure 9 Interpretation of thymine dimer template preparation and bypass replication by *in vitro* DNA replication assay....39

Figure 10 Interpretation of enzyme digestion....40

Figure 11 Defective and effective bypass replication of cyclobutane thymine dimer in XP-V and 293 cell-free extracts....46

Figure 12 Scheme of XP-V correcting factor purification procedure....47

Figure 13 High salt fraction of phosphocellulose column from 293 cell extracts complement XP-V defect on thymine dimer template with MfeI, MboI and DpnI digestion....50

Figure 14 Different amount high salt fraction of phosphocellulose column from 293 cell extracts complement XP-V defect on thymine dimer template....51

Figure 15 Sephacryl HR-200 column fraction from CFII of P-11 column complement XP-V defect on thymine dimer template....52

Figure 16 Q-Sepharose FF column fractions from CFII of P-11 appeared to have no bypass activity to complement XP-V defect on thymine dimer containing template....55

LIST OF TABLES

Table 1 NER-defect diseases: Xeroderma Pigmentosum (XP), Cockayne's syndrome (CS), Trichothiodystrophy (TTD)....7

Table 2 Comparison of Xeroderma Pigmentosum classical and variant groups....8

Table 3 Comparison the basic parameters of CFI, CFII of P-11 column fraction between 293 and Hela cell extracts....48

Table 4 Comparison of protein concentration, recovery and replication unit recovery of each peak fraction of Sephacryl HR-200 column through phosphocellulose fraction CFII....53

LIST OF ABBREVIATIONS

NER: nucleotide excision repair

MMR: mismatch repair

BER: base excision repair

XP: Xeroderma Pigmentosum

XP-V: Xeroderma Pigmentosum-Variant

CPD: cyclobutane pyrimidine dimers

T<>T: cyclobutane pyrimidine dimers

TTD: trichothiodystrophy

CS: Cockayne's syndrome

TLS: translesion synthesis

PRR: postreplication repair

RPA: Replication protein A

RFA: replication factor A

PCNA: proliferating cell nuclear antigen

TFIIH: transcription factor

Pols: DNA polymerases

α : DNA polymerases alpha

β : DNA polymerases beta

γ : DNA polymerases gamma

δ : DNA polymerases delta

ε : DNA polymerases epsilon

ζ: DNA polymerases zeta

XPA: defect in xeroderma pigmentosum group A

DTT: DL-Dithiothreitol

PMSF: phenylmethylsulfonyl fluoride

nt: nucleotide

ssDNA: single-strand circular DNA

dsDNA: double-strand circular DNA

Form I DNA:	closed circular DNA
Form II DNA:	nicked DNA
Form III DNA:	linear DNA
NP-40:	nonidet P-40

ACKNOLEDGEMENT

I wish to express my deepest gratitude to my supervisor, Dr. Daniel Lee Svoboda, for the opportunity he has provided for me to study and work on an interesting project in his laboratory, and for his helpful academic direction, patient methodological advices, and generous help in all aspects.

Special thanks are given to Dr. Bruce Stillman for providing 293 cell line, to Dr. William Kaufmann for providing XP-V cell line, to Mrs. Silvana Lachance for kind technical assistance and encouragement, to Mr. Luc Machand for collaboration on sharing the same equipment to do the experiment and computer help.

I am grateful to Dr. Claude Perreault, Dr. Elliot Drobetsky for their kind & generous assistant in providing access to laboratory space and supplies for the remaider of my study, to Dr. Johanne Tremblay, Dr. Pierre-André Lavoire for helping me in my application for a master degree program.

I would like to thank Ms. Caroline Léger, Ms. Andrea Shatilla, Dr. Jean-Christophe Hoflack, Dr. Yves paquette for their pertinent advices on my thesis, to all members of the Research Center, Maisonneuve-Rosemont Hospital for their help and courtesy to me when we worked at the same research center.

I would also like to say thanks to my family, especially my parents and my brother, my sisters for the great support, great love and the big encouragement they have provided me during my study.

DEDICATION

to my parents
to my brother
to my sisters

INTRODUCTION

I. INTRODUCTION

I.1. DNA damage

I.1.1. importance of DNA

DNA plays a key role in genetic information transmission. In order for it to remain a stable carrier of genetic information in living cells (Selby et al., 1997), DNA must be replicated completely and precisely.

I.1.2. DNA damage

DNA is attacked by endogenous and exogenous agents which cause DNA damage (Sarasin and Stary, 1997).

I.1.2.1. types and consequences of DNA damage

I.1.2.1.1. types of DNA damage

There are at least two major classes of DNA damage: spontaneous alterations of DNA bases, and enviromental damage to DNA. DNA damage can be caused by sunlight and enviromental pollutants. Ionizing radiation such as X-rays causes base damage, sugar damage and strand breaks. Ultraviolet light (UV) at 254nm induces cyclobutane pyrimidine dimers (CPD), pyrimidine-pyrimidone (6-4) photoproducts (figure 1). Numerous chemical agents cause interstrand cross-links and mono-adducts; cisplatin, psoralens, benzo[a]-pyrene induce a variety of lesions that interfere with the proper functioning of DNA (Friedberg et al., 1994; Hoeijmakers, 1993).

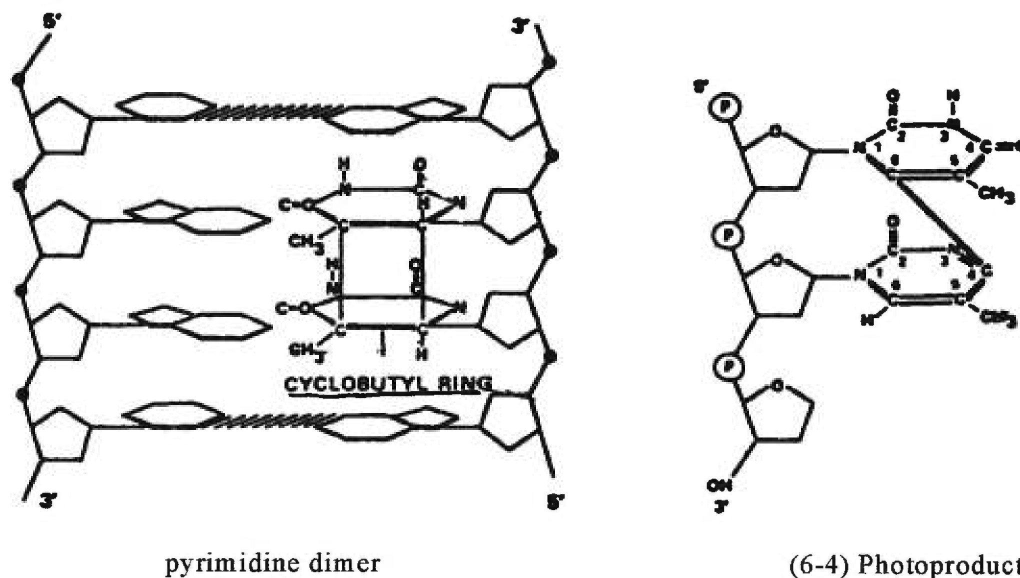

Figure 1. structure of cyclobutane pyrimidine dimer and pyrimidine-pyrimidone (6- 4) photoproduct by UV irradiation. The cyclobutane pyrimidine dimer is formed in DNA by covalent interaction of the 5, 6 double bonds of two adjacent pyrimidines in the same poly nucleotide chain.(6-4) photoproduct is produced by linkage between the C-6 position of one thymine and the C-4 position of the adjacent thymine.

I.1.2.1.2. consequences of DNA damage

As well as its immediate hampering effect on vital processes, notably transcription and replication, DNA damage may also give rise to mutations and chromosomal aberrations in the absence of efficient error-free repair via replication of DNA damage. These nucleotide sequence changes lead to inborn defects, carcinogenesis and cell death. The time-dependent accumulation of DNA damage and mutations may even contribute to (cellular) ageing (Sarasin and Stary, 1997; Hoeijmakers, 1993) (figure2).

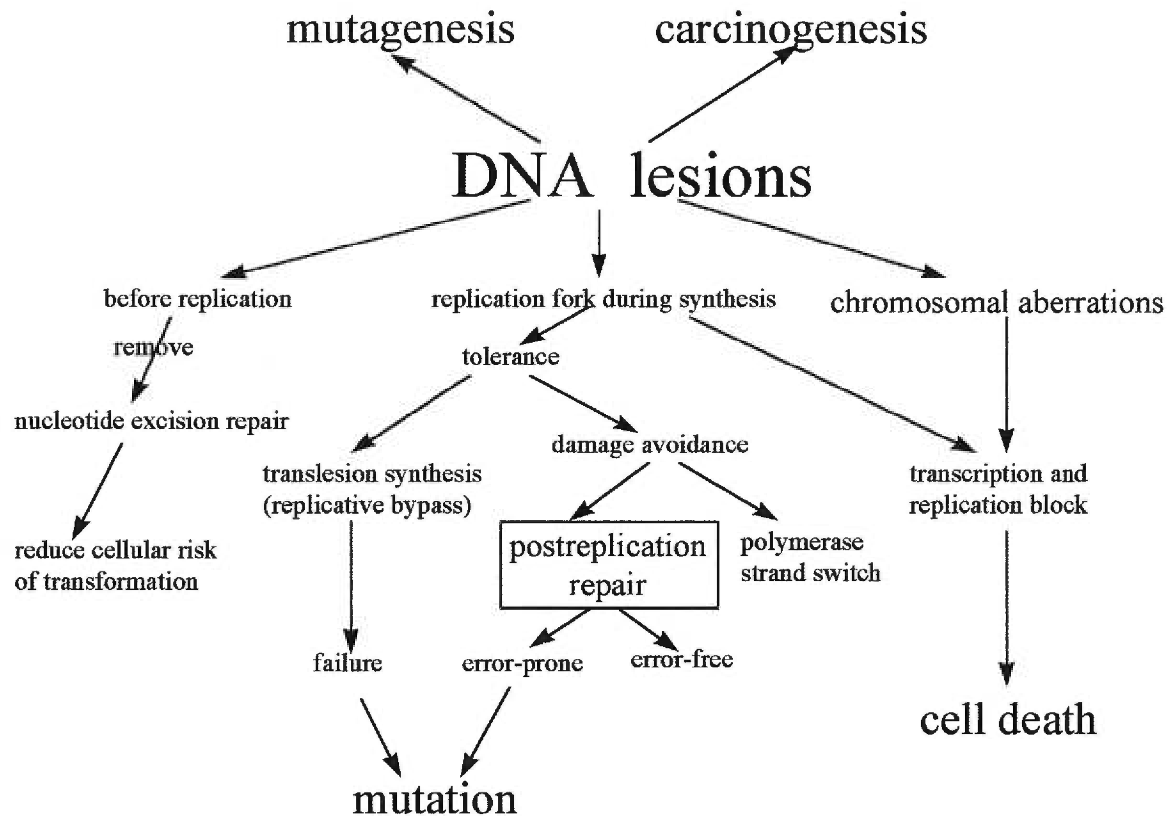

Figure 2. Consequences of DNA damage. DNA damage causes mutagenesis, carcinogenesis, and cell death.

I.2. DNA repair

I.2.1. pathways of DNA repair

Living organisms have developed various strategies which act as a kind of intranuclear 'immune system' that is able to recognize and eliminate many types of lesions. These DNA repair systems prevent the genome from the damaging effect of endogenous and exogenous mutagens by removing them and restoring the normal nucleotide sequence (Friedberg et al., 1994). DNA repair pathways are well conserved from bacteria to man: nucleotide excision repair (NER), mismatch repair (MMR) and base excision

repair (BER) (Figure 3).These repair systems remove 27-29 nucleotide oligomers,300-500 nucleotide patches, and 1-4 nucleotides, respectively (Sancar, 1995).

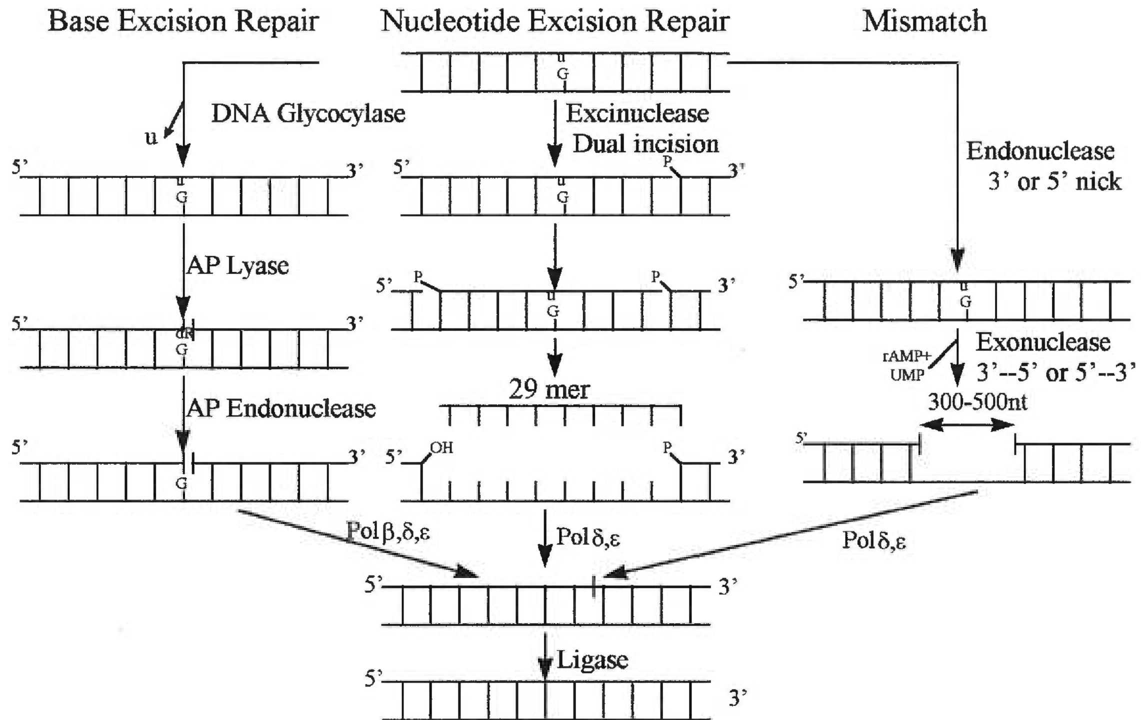

Figure 3. DNA repair pathways: base excision repair, nucleotide excision repair and mismatch repair.

I.2.2. NER is the most frequent pathway for the removal of DNA damage

The most important and frequent repair pathway is NER It recognizes and removes almost all types of DNA lesions including various UV-induced photoproducts (cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts), chemical adducts and certain types of crosslinks (Sarasin and Stary, 1997).

I.2.2.1. NER and UV radiation

UV irradation is subdivided into three regions: UVA (315-400 nm),UVB (280-315 nm), and UVC (200-280 nm) (figure 4).UVC is comprised of the most potent wavelengths for inducing DNA damage. The major types of damage induced by UVC are cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts. These are formed by the direct absorption of UVC by DNA at dipyrimidine sites and constitute approximately 70-80 % and 20-30 % of UVC-induced DNA damage,respectively (Clingen et al., 1995). The most frequent UV photoproduct is T<>T, which has been characterized

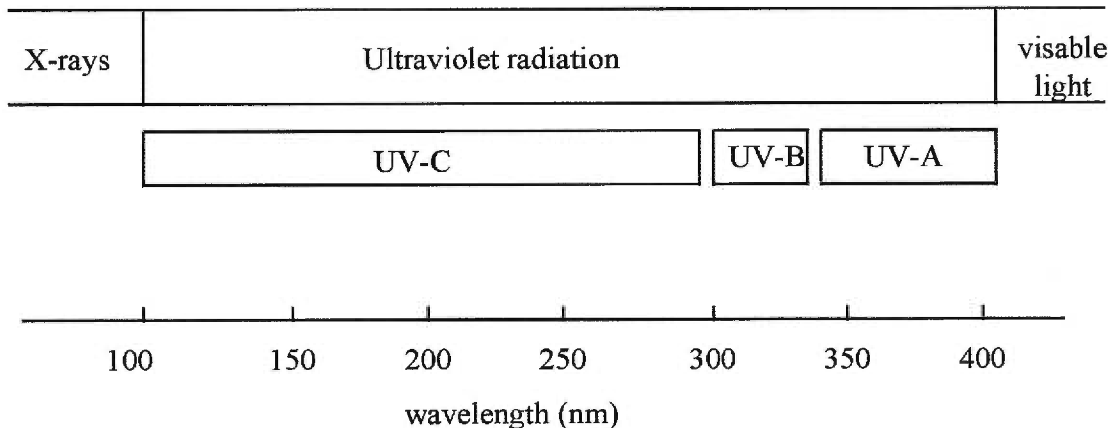

Figure 4. UV radiation spectrum.

extensively by physical (Wang, 1976), and enzymatic (Friedberg, 1985) methods due to its stability (Kunieda and Witkop, 1971), and convenient preparation (Taylor and Brokie, 1988). It is removed by the enzymatic process NER (Wang, 1976; Setlow, 1966).

I.2.2.2. Enzymatic mechanism of NER in *E. coli*

In all organisms, NER consists of five steps: damage recognition, dual incision of the damaged strand flanking the lesion at some distance from it, excision of the lesion-containing oligonucleotide (dual incision), synthesis of new DNA using undamaged strand as template,and ligation. The reaction is in principle error-free (Hoeijmarkers,1993;Sancar, 1995). It is well understood in *E. coli* in which at least six structural genes (UvrA, UvrB, UvrC, UvrD, polA and lig) and two regulatory genes (recA and lexA) are involved. The crucial step is the detection of a lesion by discrimination between abnormal and normal

DNA structures. It is carried out by the cooperative action of two proteins: UvrA and B. The A_2B complex is able to recognize an impressive spectrum of damaged structures ranging from thymine glycols to bulky chemical adducts and inter- and intra-stand crosslinks. Following damage recognition, UvrC joins the complex, and dual endo-nucleolytic breaks are introduced into the DNA, seven nucleotides 5' and three to four nucleotides 3' to damage site, regardless of the nature of the lesion. This mechanism is highly efficient, removing these lesions which are usually present in trace amounts (concentrations of 1 in 10^6 nucleotides or less) (Hoeijmakers, 1993; Grossman and Thiagalingam, 1993).

I.3. NER-defect diseases

The mechanisms of NER in human cells are rapidly gaining clairity (Cleaver, 1968; Setlow et al., 1969;Buhl and Regan, 1973;Meneghini, 1976; Lehmann and Kirk-Bell,1978; Fornace, 1983; Wang et al., 1993; Aboussekhra and Wood, 1994; Sancar, 1996). The phenotypic consequences of defective NER in humans are apparent in rare but dramatic diseases characterized by hypersensitivity to UV and a striking clinical and genetic heterogeneity. The xeroderma pigmentosum syndrome (XP), Cockayne's syndrome (CS), and the photosensitive form of Trichothiodystrophy (TTD) are three of these clinically distinct human disorders inherited as an autosomal recessive trait.

I.3.1. CS and TTD

The direct link of defective DNA repair to cancer seems to be complex, since in contrast to patients with XP, those with TTD or CS do not have an increased frequency of skin cancers. The understanding of the absence of skin tumors in TTD and CS patients may offer a way to better protect normal individuals from the most rapidly increasing cancer: skin cancer (Sarasin and Stary, 1997) (table 1).

Disease(s)	Associated syndrome	skin cancer	UV sensitivity
XP	XP/XP,CS, XP/CS/TTD	+	+++
CS	CS	-	+
TTD	TTD	-	+

Table 1. NER-defect diseases: Xeroderma Pigmentosum (XP), Cockayne's syndrome (CS), Trichothiodystrophy (TTD).

I.3.2. XP complementation groups

XP is an autosomal recessive disorder characterized by extreme sensitivity to sunlight, as manifested by erythema, freckles, xerosis, and scaling of the skin. XP individuals also exhibit the hallmark symptom of a DNA-damage processing disease, i.e., "a marked predisposition to skin cancers developing after exposure to sunlight", involving a "unique conjunction of enviromental, genetic, and biochemical factors in the etiology of cancer" (Cleaver and Kraemer, 1989). XP affects between 1 in 40,000 and 1 in 200,000 individuals worldwide (Misra and Vos, 1993). They develop skin cancer at a 2000-fold higher rate than normal (Cleaver and Kraemer,1989; Kraemer et al.,1984,1987) in sunlight exposed skin areas (Misra and Vos, 1993; Bredberg et al., 1986). They also have various defects in cellular immunity (Jimbo et al.,1992). Complementation tests by cell fusion have provided evidence for the existence of seven genetic complementation groups (A-G) of "classical" XP (Robbins, 1988; De Weerd-Kastelein et al., 1972; Kraemer et al.,1975;Arase

et al.,1979; Keijzer et al., 1979), corresponding to defects in each of seven different genes required for nucleotide excision repair (NER; Fornace et al., 1976; Friedberg et al., 1977) which results in a failure to repair DNA damage from UV light and sunlight (Hessel et al., 1992; Seguin et al.,1992). An eighth "variant" group (XP-variant, XP-V), fails to replicate UV-damaged DNA correctly (Hessel et al., 1992).

I.4. XP-V

XP-V patients show the clinical characteristics of the disease, are predisposed to skin cancer with high incidence (Seguin et al., 1992; Boyer et al., 1990), although the average age of onset for XP-V is later than in classical XP; i.e. 15 and 43 years respectively (Friedberg et al., 1994; Cleaver and Kraemer,1989; Fujiwara et al.,1987). In contrast to the other forms of XP, XP-V cells have a normal, or nearly normal rate of nucleotide excision repair of UV-induced DNA damage (Wang et al., 1993; Burk et al., 1971; Cleaver, 1972; Cleaver and Thomas, 1969), and a nearly normal rate of post-UV survival (Wang et al., 1993; Watanabe et al., 1985; Lehmann, 1972) (table 2). They are uniquely characterized by an abnormality in the manner in which DNA replicates on templates containing UV and other lesions (Lehman et al.,1975; Leaver et al., 1979; Boyer et al., 1990).

	Classical XP	XP-V
UV sensitivity	+++	+
skin cancer	+	+
age (normal 58 years old)	<16 years old	43 years old
defect in NER	+	-
defect in PRR	-	+

Table 2. Comparison of Xeroderma Pigmentosum classical and variant groups.

I.4.1. experimental evidence of XP-V

Investigations of XP-V indicate aberrant DNA replication following treatment with DNA damaging agents (Lehmann, 1972). XP-V cells require a highly protracted period to achieve full length DNA synthesis after UV (Cleaver, 1968; Lehman, 1975). Indeed, it is estimated that XP-V has ~25-33 % of normal UV-damage replication capacity (Boyer et al., 1990). Since DNA repair (Burk et al.,1971; Cleaver,1972; Cleaver and Thomas, 1969), and UV-inhibition of replicon initiation (Park and Cleaver, 1979; Kaufmann et al., 1980; Kaufmann and Cleaver, 1981) are both normal in XP-V, a defect in elongation of replication intermediates at DNA damage sites has been proposed (Kaufmann and Cleaver, 1981; Cleaver, 1979; Watanabe et al., 1985). Agents other than 254 nm UV have been examined as well. Psoralen plus UVA (Cimino et al., 1985) produces an abnormal reduction in replication in XP-V cells (Misra and Vos, 1993). In contrast, adducts induced by benzo[a]pyrene-diol-epoxide, a carcinogenic metabolite derived from tar, present in tobacco smoke (BPSE-I; Hall, 1990; Selkirk et al., 1982), create such an effective block to replication in normal cells that differential inhibition of replication between normal and XP-V is not observed (Boyer et al.,1990; Cordeiro-Stone et al.,1986).XP-V patients are also predisposed to skin cancer, and cells are hypersensitive to the genotoxic effects of UV light (Maher et al., 1976; Boyer et al., 1990). However, they are significantly more sensitive to its mutagenic effect (Wang et al., 1991).

I.4.2. discovery of XP-V mutagenesis by shuttle vector

A shuttle vector carrying the *supF* gene of *E. coli* as genetic target was employed to probe the mechanism of UV mutagenesis in XP-V cells (Wang et al., 1991, 1993). The frequency of mutants increased linearly with dose, but with a slope 5 times steeper than that seen with normal cells (Bredberg et al., 1986; Wang et al., 1991, 1993; Waters et al., 1993).In addition,the spectrum of UV-induced mutations in XP-V cells consisted primarily

of transversions at A:T base pairs(73%),which differed markedly from the high proportion of G:C→A:T transitions at G:C base pairs found in normal cells (60%)(Wang et al.,1991). In fact, a high proportion of G:C→ A:T transitions is a virtually universal characteristic of UV-irradiated cells. This has been attributed to the so-called "A-rule", which is named for the observation that during UV mutagenesis, adenine is preferentially incorporated opposite non- coding lesions like dimerized pyrimidine bases (Strauss, 1991). Also interesting is the recovery of a unique transversion mutation hotspot in the *supF* gene at the central nucleotide in a run of three T's. The most likely premutagenic lesion at this site is the T<>T cyclobutane dimer (Setlow, 1966). This DNA photoproduct is only poorly mutagenic in normal human cells (Bredberg et al., 1986; Glazer et al., 1986; Hauser et al., 1986; Protic-Sabljic et al., 1986; Brash et al., 1987; Brourre et al., 1989; Drobetsky et al., 1989; Hsia et al., 1989), thereby providing a potential signature mutation for the XP-V phenotype.The mutational specificity of UV light in XP-V cells is consistent with deviation from the "A-rule," in agreement with the hypothesis that XP-V gene product influences nucleotide insertion at damage sites in the DNA template. This could count for the hyper-mutability of XP variant cells after UV irradiation with increased skin cancer susceptibility (Lehmann, 1974; Wang et al., 1991; Strauss, 1991).

I.4.3. unclear mechanism of describing XP-V

The evidence discussed so far points to an XP-V defect at the level of translesion synthesis (TLS). This is one of the lesion tolerance strategies in which lesions persist at the replication fork during DNA synthesis. It is a process during which the replication machinery reads through the lesion with an associated risk of fixing a mutation. If the lesion does not impede DNA synthesis, an unmodified replication complex can achieve TLS (figure 2). Another lesion tolerance strategy is damage avoidance, which facilitates replication of damaged DNA templates without the need for the polymerase to read through the lesion. This strategy takes advantage of the information contained in the complementary strand (Napolitano et al., 1997). Two models of damage avoidance have been proposed in *E. coli* (Rupp et al., 1971; Higgins et al., 1976; Livneh et al., 1993):

postreplication recombinational repair and polymerase strand switching. In the former, the DNA polymerase blocked at a lesion site dissociates from the DNA and reinitiates replication downstream from the lesion, leaving a gap that is repaired by a recombination mechanism involving the sister chromatid. In the latter, DNA polymerase switches temporarily from the damaged parental template to the undamaged newly synthesized strand of the sister chromatid before returning to the parental emplate down-stream from the lesion. Both damage avoidance models are believed to be efficient and error-free in *E. coli* (Napolitano et al., 1997).

I.5. relationship of XP-V and postreplication repair (PRR)

I.5.1. definition of PRR

PRR may be defined as the elimination of discontinuities in the daughter-strand DNA and the replicative bypass of lesions in the DNA template (Boyer et al., 1990). Actually PRR is not a real “repair” process, since the damage as such is not removed (Lehmann, 1974). Pathways of PRR reduce the number of DNA growing points that are blocked at template lesions and increase the rate of growth of nascent DNA on damaged templates (Boyer et al., 1990).

I.5.2. possible mechanism of PRR

Several hypotheses about the mechanisms of PRR are translesion synthesis, template switching and recombinational repair.

I.5.2.1. translesion synthesis

Translesion synthesis (gap filling model, de novo syntheses; Lehamann, 1972) is now the most accepted mechanism to describe XP-V defect after UV irradiation. It was first proposed by Lehmann (1972). According to this model (Figure 5): (a) DNA synthesis

continues until it reaches a thymine dimer in parent strands, (b) gaps are left in newly-synthesized daughter DNA strands opposite dimers during replication, (c) gaps are then filled in by de novo synthesis. Lesions in DNA could affect the growth of daughter-strands (Lehmann, 1974). If lesions completely block DNA replication (a), DNA synthesis may continue around lesion, with gaps left opposite lesions and filled in some time later (b) or very rapidly (c). Alternatively, DNA synthesis continues past lesions(d) (figure 6).

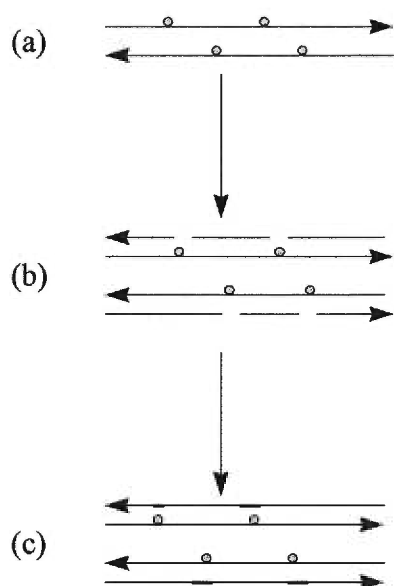

Figure 5

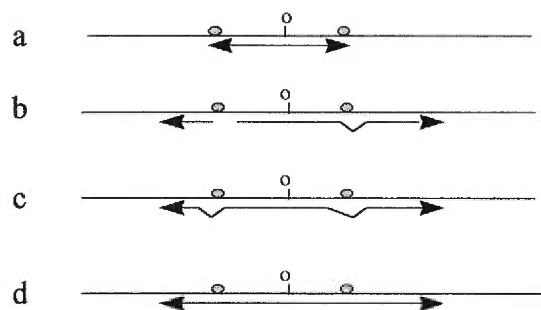

Figure 6

Figure 5. Model of postreplication repair—translesion synthesis. DNA synthesis is blocked by thymine dimer, leaving gaps in newly-synthesized daughter DNA strands opposite the dimers. Gaps are filled in by de novo synthesis.

Figure 6. Models for replication on damaged templates. (a) DNA synthesis may be halted completely. (b) It may be delayed and then continues beyond the lesions leaving a discontinuity opposite the lesion, and sealed some time later. (c) as (b), expect discontinuity may be sealed very shortly after its formation. (d) DNA synthesis continues around the lesions.

Rupp and Howard-Flanders (1968) found that the DNA synthesized in UV-irradiated cells had a lower molecular weight (as measured in alkaline sucrose gradients) than that from unirradiated cells, suggesting that this newly synthesized DNA contained gaps. DNA synthesis after ultraviolet irradiation proceeds in human normal (WI38) cells then gaps are formed along the newly made strand,leaving regions of single strandness in template DNA. As replication proceeds these gaps disappear and 2 h after irradiation (100-250ergs/mm^2) they are barely detected by the endonuclease assay (Meneghini, 1976). Subsequently the gaps are filled in, as demonstrated by an increase in size of the newly synthesized material during further incubation (Lehmann, 1972). Rupp and co-workers presented data which suggested that the gaps in the new strands were opposite pyrimidine dimers in the parental strands (Howard-Flanders et al.,1968) and estimated that the size of the gaps was about 1000 to 1600 nucleotides in length by poisson distribution analysis of the percentage of the L-DNA produced as a function of S1 nuclease concentration (Iyer and Rupp, 1971; Meneghini et al., 1981). The size of short pieces of newly- synthesized DNA is approximately equal to the average distance between dimers in the parental strands, as calculated from the fraction of thymine in pyrimidine dimers (Lehmann, 1972; Buhl et al., 1972; Zipser, 1973). But these are controversial results, favoring either the continuous or the discontinuous mechanism of DNA synthesis after irradiation (Painter, 1975). If the dimers merely delay DNA chain elongation there is no need for some unique process for repairing daughter DNA strands in irradiated cells.

I.5.2.2. template switching

With template switching or strand displacement (Higgins et al., 1976), the principle feature is the use of a newly synthesized sister strand as an alternate template for the replication of a damaged region in the homologous parental strand. Normal replicative synthesis proceeds until blocked by a lesion on one template strand, thereby providing a detour around a lesion, which is not recognized as a functional template. Reassociation of parental strands by displacement of newly synthesized DNA (branch migration) permits

pairing of the two daughter strands and provides an alternate template for synthesis (Figure 7). The copying of the sequences complementary to the damaged region bridges the block to replication, and allows synthesis to proceed without necessitating the removal of the initial lesion. There is free 3' OH group to serve as primer on the alternate template, and requires some unspecified factor to open up the helix at the growing point (Higgins et al., 1976). No experimental results exist that are in obvious support of this model. It does not involve replication "gaps" and makes use of the known processes of strand displacement and branch migration (Lee et al., 1970; Masamune and Richardson, 1971; Broker and Lehmann,1971). However, Lavin (1978) thinks this strand displacement model does not provide a satisfactory mechanism to account for the dissociation of both newly synthesized strands of DNA in the region of the damage, their associate with one another, the subsequent dissociation of these strands,and their re-annealing in the original duplexes.

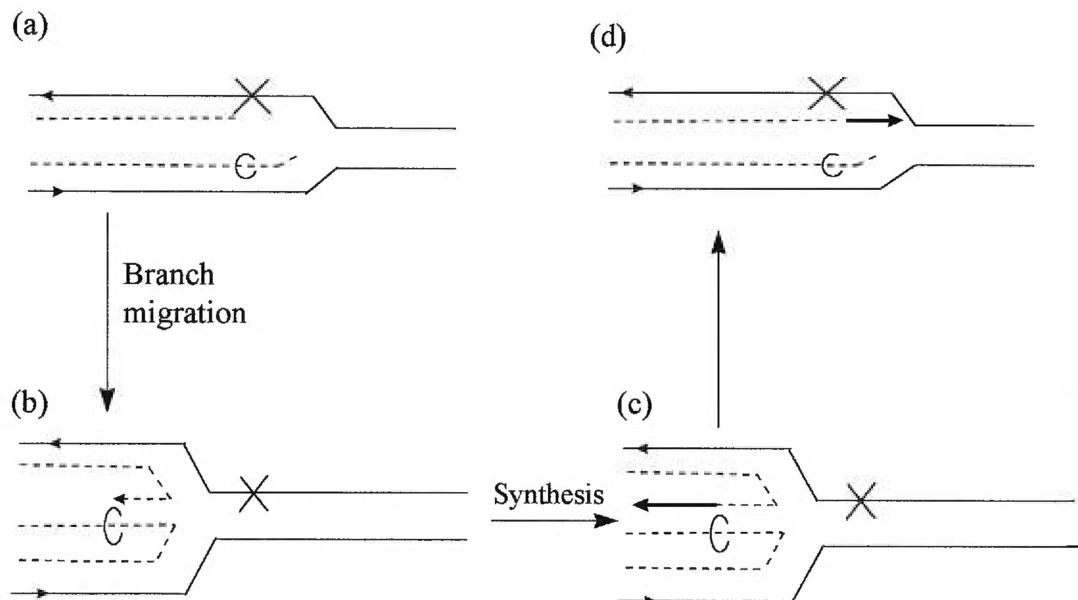

Figure 7. Model of postreplication repair—template switching. Strand displacement and branch migration create an alternate template allowing replication to bypass a lesion (X).

I.5.2.3. recombinational repair

Recombinational repair (Ganesan,1974; Rupp and Howard-Flanders, 1968) (figure 8): DNA replication proceeds until it reaches a dimer, where the DNA polymerase stops and skips over the dimer leaving a gap of ~103 nucleotides in the newly synthesized DNA (Buhl and Regan, 1974).These gaps are subsequently sealed, filled in by the corresponding piece of parental DNA from the sister duplex (Rupp and Howard-Flanders, 1968) and the lost genetic information regained by a process involving recombinational exchanges between sister duplexes (Rupp et al., 1971). The resulting structure contains end-to-end associations of parental and daughter strand DNA (Rupp et al., 1971), with pyrimidine dimers equally distributed between parental and daughter strands (Ganesan, 1974). But Lavin (1978) proposed a model for recombinational repair in mammalian cells. This model involves the introduction of a single-strand interruption into one of the parental DNA

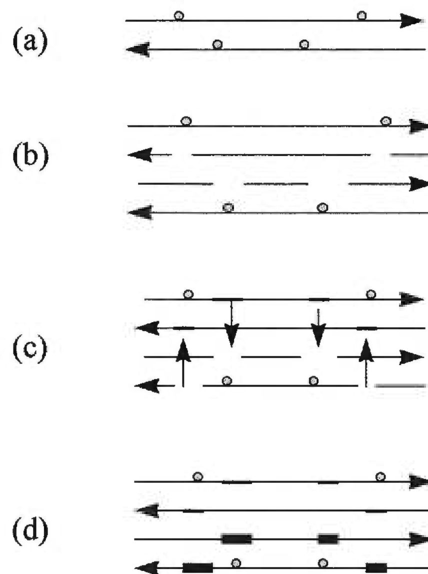

Figure 8. Model of postreplication repair—recombinational repair. Synthesis of DNA is interupted by thymine dimer. Incision occurs on the complementary undamaged parental strand, followed by strand migration and reciprocal crossing-over. The overall effect is the bypassing of the damaged site.

strands, followed by strand crossing-over (Broker and Lehmann, 1971; Radding, 1973; Holliday,1974; Meselson and Radding,1975), as means of circumventing the damage. UV irradiation gives rise to pyrimidine dimers in DNA, gaps are formed opposite pyrimidine dimers or other forms of base damage after DNA replication. Then, a nick is introduced into the complementary parental strand, and the nicked strand of a duplex molecule switches to pair with the other parental chain by strand migration. Reciprocal crossing over involving polymerization at the 3' end of the interrupted daughter strand follows, then the linkage point (crossing-over point) is free to migrate in either direction. As the linkage point moves to the right, restoration of the original helices occurs. The overall effect is closure of the discontinuity opposite the lesion in DNA without the exchange of DNA strands or the requirement for a polymerase activity to read over the damaged region. It seemed consistent with available information on postreplication repair of UV damage (Lavin, 1978). This is the first proposed mechanism for recombinational repair in humans.

I.5.3. the mechanism to elucidate XP-V defect in NER is still unclear

None of these mechanisms can satisfactly explain the defect in XP-V cells. Studies aimed at elucidating the mechanism of DNA synthesis on damaged DNA templates *in vivo* have yielded controversial results (Buhl and Regan, 1973). The precise series of molecular events beginning with replication forks encountering damaged nucleotides, and culminating in a fully replicated genome, have not been clearly elucidated in human cells. On the other hand, with *E. coli* as a model organism, it has been clearly shown that daughter strand gap repair (error-free gap filling, post-replication repair, PRR), employing homologous strand exchange from the sister chromatid, largely accounts for the replication of the UV-damaged genome (Smith and Menu, 1970; Rupp et al., 1971; Ley,

1973; Sedgwick, 1975; Youngs and Smith, 1976). Under SOS conditions, however, error-prone translesion synthesis makes a significant contribution (Bridges and Mottershead, 1976; Bridges and Bates, 1990; Hagensee et al., 1987). Since recombinational repair of daughter strand gaps in human cells remains an unresolved issue (Buhl and Regan, 1973; Higgins et al., 1976; Meneghini,1976; Lehmann and Kirk-Bell, 1978; Fornace, 1983), any conclusions about such a mechanism in XP-V cells have been thus far precluded.

I.5.3.1. experimental evidence of XP-V defect

Rude et al. (1975) measured rates of DNA synthesis and strand growth in solvent- and carcinogen-treated cells by velocity sedimentation analyses of radiolabeled nascent DNA in alkaline sucrose gradients, and found that the molecular weight of newly synthesized DNA from the XP variants is much lower than that from normal cells after a fluence of 8 J/m^2 of UVC 254 nm. XP-variants have a slow recovery of DNA synthesis after irradiation with ultraviolet light, and caffeine enhances their sensitivity (Buhl and Regan, 1974). These data strongly suggest that persistant gaps are left in the daughter strands in XP variants under conditions in which gaps, if they are formed at all in normal cells, are very transient (Lehmann et al.,1977). XP variant cells express maximally 25-33% of the total PRR activity observed in normal fibroblasts after UV treatment (Boyer et al., 1990). Replication segments were significantly shorter in UV-exposed XP variant cells than in UV-exposed wild-type human cells immediately after exposure. There is more blockage of DNA fork progression at UV-induced lesions in XP variant cells than in normal cells. With time, (2.5-5.0 h) the lengths of replication segments increased in both cell lines, suggesting that some type of bypass was occurring in XP variant cells or that excision repair was removing the blocking lesions (Griffiths and Ling,1991). Conceivably, this deficiency in PRR activity results in the XP variant's increased risk of cancers induced by sunlight, because XP variant cells and normal fibroblasts are equally proficient in excision repair (Boyer et al., 1990). Human cells prelabeled with [^{32}P] phosphate were exposed to UV and then pulse-labeded with [^{3}H] thymidine. The DNA from these cells was subsequently treated with T_4 endonuclease V. Sedimentation in neutral sucrose

gradients showed that the molecular weight of native DNA remained unchanged for both DNAs upon endonuclease treatment (which specifically nicks DNA at positions adjacent to pyrimidine dimers),indicating that gaps opposite dimers are not necessarily formed after irradiation (Meneghini and Hanawalt, 1975).

I.5.3.2. requirement of the approaches to detect XP-V defect

Based on the experimental evidences and results on XP-V studies, new approaches to detect the XP-V defect must be employed to elucidate the biochemical and molecular biological characteristics of this cancer-prone disease. The successful elucidation of nucleotide excision repair and classical XP will be helpful in understanding of XPV (Wood et al., 1988; Silbghatullah et al., 1989; Reardon et al., 1991; Szymkowski et al., 1993). In fact, by *in vitro* complementation of XP cell extracts, in addition to a classical resolution and reconstitution approach, it has been possible to purify classical XP correcting factors involved in nucleotide excision repair in humans (Aboussekhra et al., 1995; Mu et al., 1995). The *in vitro* methods give us a basic and comprehensive understanding of the molecular mechanism of nucleotide excision repair in humans (Sancar, 1995; Wood, 1995).

I.6. *in vitro* DNA replication assay

I.6.1. SV40 DNA replication assay and the importance of T antigen

An *in vitro* SV40-based DNA replication assay has been utilized to demonstrate the XP-V defect (Svoboda et al., 1998). Simian virus 40 (SV40) is a simple and excellent model system for a single chromosomal replicon that has proven useful for studying the mechanisms of DNA replication in animal cells (Challberg and Kelly, 1982; DePamphilis and Wassarman, 1980, 1982; Li and Kelly, 1984). The SV40 genome is a double-stranded circular DNA molecule containing 5,243 base pairs (bp) (Reddy et al.,1979; Cremisi et al., 1976). In infected cells, SV40 genome is complexed with histones to form a mini-

chromosome with a nucleoprotein structure analogous to that of cellular chromatin (Cremisi et al., 1976; Griffith, 1975). DNA replication is initiated within a unique origin on the viral chromosome and the elongation of nascent DNA chains proceeds bidirectly (Danna and Nathans, 1972; Fareed et al., 1972). The minimal origin is located within a 65-base-pair segment of the viral genome and contains a high-affinity recognition site for the SV40 T antigen (site II) (Li et al., 1986). The initiation reaction requires a specific interaction between the origin sequence and virus-encoded initiation protein, large tumor (T) antigen (Danna and Nathans, 1972; Fareed et al., 1972; Chou et al., 1974; Hay and DePamphilis, 1982). This is the only viral protein with multifunction which is involved in cellular transformation and plays a central role in DNA replication (Kelly et al.,1988; Melendy and Stillman, 1991). It is a sequence-specific DNA binding protein and a DNA helicase. T antigen specifically binds to the viral origin of SV40 and initiate the DNA replication. The initiation process results in establishment of two replication forks that move in opposite directions from the origin (Li et al., 1986; Wold et al., 1989). Except the virus-encoded large T antigen, all the proteins required for SV40 DNA systems are supplied by the host cell (Wold et al., 1989; Hurwitz et al., 1990).

I.6.2. proteins of reconstitute system by *in vitro* assay

A cell-free system has been developed which mimics SV40 replication *in vivo* and demonstrates the same TAg and SV40 origin dependence seen in genetic experiments. It is capable of replicating plasmid DNA templates containing the SV40 origin of DNA replication (Li and Kelly, 1984; Stillman and Gluzman, 1985), since cytoplasmic extracts prepared from SV40-infected or -uninfected monkey cells supplemented with purified SV40 T antigen can efficiently replicate DNA templates containing a functional origin of DNA replication (Stillman and Gluzman, 1985). The *in vitro* system has proven amenable to biochemical analysis, and fractionation of human cell extracts has lead to the identification of at least 30 polypeptides that are essential for complete SV40 DNA replication *in vivo* (Melendy and Stillman,1991;Li et al.,1993). These proteins are involved

in initiation and elongation of DNA chains (Stillman,1983; Kelly,1984): replication factor A (RPA), proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), etc.

I.6.2.1. replication protein A

Replication protein A [RPA; also known as replication factor A (RFA), or human single-stranded DNA-binding protein (hSSB)] plays an essential role in DNA replication, early step of nucleotide excision repair, and homologous DNA recombination (Pan et al., 1995). RPA is a stable complex of three polypeptides of 70, 34 and 11 kDa (Wold et al., 1989; Brill and Stillman, 1989; Matsuda et al, 1995; Wold,1997). The largest subunit (70 KDa) contains an evolutionarily conserved zinc finger motif (RPAm) that lies outside of the domains required for binding to single-stranded DNA or forming the RPA holocomplex. RPAm associates with T antigen, assists the unwinding of double-stranded DNA at an origin of replication, stimulates DNA polymerases alpha and delta, and supports the formation of the initial short Okazaki fragments. The phosphorylation of p34 is catalyzed by both cyclin-dependent kinase- cyclin A complex and DNA-dependent protein kinase (Pan et al., 1995). RPA binds to another protein XPA, to recognize the damaged sites (Wood,1997).Members of this family bind nonspecifically to single-stranded DNA and interact with and/or modify the activities of multiple proteins. In cells, RPA is phosphorylated by DNA-dependent protein kinase when it is bound to single-stranded DNA (during S phase and after DNA damage). Phosphorylated RPA may play a role in coordinating DNA metabolism in the cell. RPA may also have a role in modulating gene expression (Wold, 1997; Lin et al., 1998). Although other SSBs can replace RFA for unwinding, the requirement for RFA is very specific in that no other SSB tested is able to replace RFA for the elongation stages of DNA replication (Wold and Kelly, 1988; Kenny et al., 1989).

I.6.2.2. proliferating cell nuclear antigen

Proliferating cell nuclear antigen (PCNA) is a component of protein complexes containing DNA replication, repair and cell cycle regulatory proteins (Loor et al., 1997) present in the cell nucleus at locations that correspond to the sites of DNA synthesis (Bravo et al., 1985; Madsen and Celis,1985). It is essential for DNA polymerase δ (pol δ) activity, acting as an auxiliary factor in the pol δ elongation reaction (Hurwitz et al.,1990), greatly increasing the processivity of purified pol δ (Bravo et al., 1987). It is also required for synthesis of the leading DNA strand and in its absence only DNA products from the lagging strand template are observed (Prelich et al., 1987a, 1987b; Prelinch and Stillman, 1988). Its mechanism is that of a molecular clamp, reducing the rate of polymerase δ dissociation from the primer end therefore increasing its processivity (Svoboda et al., 1998).

I.6.2.3. replication factor C

Replication factor C (RFC) is a five subunit complex including p140, p40, p38, p37, and p36 which acts as a processivity factor for eukaryotic DNA polymerases delta and epsilon, increases affinity of pol δ for primer ends and reduces PCNA requirement in pol δ reaction (Hurwitz et al., 1990; Uhlmann et al., 1997). RFC binds to a DNA primer end and loads PCNA onto DNA in an ATP-dependent reaction. The N-terminal region of the small subunits plays a critical role in the function of these subunits, deletion of which reduces but does not abolish RFC activity in loading PCNA on DNA and in supporting an RFC-dependent replication reaction. The N termini of p37 and p40, although highly homologous, are not inter-changeable, suggesting unique functions for the individual subunits (Uhlmannet al., 1997). RF-C is a primer recognition protein with an associated DNA-dependent ATPase activity (Tsurimoto and Stillman, 1990, 1991a, 1991b).

I.6.2.4. XPA

XPA is a zinc finger DNA-binding protein, which is missing or altered in group A xeroderma pigmentosum cells. It preferentially binds damaged and single-stranded DNA,

associate with the RPA and is known to be involved in the damage-recognition step of the NER processes (Wood, 1997; Matsuda et al., 1995).

I.6.2.5. transcription factor

Transcription factor (TFIIH) is a general transcription factor for RNA polymerase II that in addition is involved in DNA excision repair, composed of XPB, XPD, p62, p52, p44, p34 (form the core TFIIH), cdk7, cyclin H, MAT1 nine subunits (Wood, 1997; Rossignol et al., 1997), direct the transcription (Wood, 1997), exhibit helicase/ATPase, kinase activity and DNA binding functions (Rossignol et al., 1997; Léveillard et al., 1996), regulate cell cycle (Bhatia et al., 1996; Seroz et al., 1995). It is responsible for unwinding DNA and releasing the excised fragment from the complementary strand (Hoeijmarkers and Bootsma, 1994).

I.6.2.6. DNA polymerase

DNA polymerases (Pol) are involved in replication, repair and recombination. At least six different Pols are known,named alpha (α), beta (β), gamma (γ), delta (δ), epsilon (ε), and zeta (ζ). Among them, Pol delta occupies important roles in DNA replication, nucleotide excision repair, base excision repair and V(D)J recombination. Pol α is extremely conserved in evolution from yeast to man (Hindges and Hubscher, 1997). A switch from the initiation to polymerase δ occurs through the recognition of free DNA 3'-ends by a complex of RF-C and PCNA (Tsurimoto and Stillmanm, 1991a, 1991b; Lee et al., 1991), two protein complexes that build together the moving platform for Pol delta. This moving platform provides an important framework for dynamic properties of an accurate Pol delta such as its recruitment when its function is needed, the facilitation of Pol delta binding to the primer terminus, the increase in Pol delta processivity, the prevention of non-productive binding of the Pol delta to single-stranded DNA, the release of Pol delta after DNA synthesis and the bridging of Pol delta interactions to other replication proteins (Hindges and Hubscher, 1997). DNA polymerase δ then performs

highly processive elongation of leading and lagging strands in conjunction with PCNA (Tsurimoto and Stillmanm,1991a,1991b;Lee et al.,1991). Polymerase α/primase continues to perform lagging strand initiation as the replication fork progresses (Tsurimoto et al., 1990; Weinberg et al., 1990; Eki et al., 1992). Likewise, DNA polymerase beta was able to elongate the arrested replication products of the other three DNA polymerases, thus showing its capacity to successfully compete with poly-merase alpha, delta, and epsilon in the stalled replication complex (Hoffmann et al., 1995). DNA polymerase ε is required for viability (Araki et al.,1992), and may be involved in addition to polymerase δ in leading and/or lagging strand elongation (Burgers, 1991; Podust and Hubscher, 1993).

I.6.2.7. topoisomerases

Topoisomerase I manages superhelicity ahead of the replication fork (Sundin et al., 1981). Maturation and ligation of Okazaki fragments ensues through the action of RNaseH/MF1 exonuclease and DNA ligase I (Waga et al.,1994;Goulian et al.,1990; Ishimi et al.,1988; Turchi and Bambara,1993). Topoisomerase II effects decatenation of daughter molecules (Ishimi et al., 1988).

I.6.2.8. XPC-hHR23B

XP group C (XP-C) is one of the most common complementation groups, only defective in the global genome repair subpathway of NER and proficient in the removal of lesions from the transcribed strand of active genes (Venema et al., 1991). XP-C protein is involved in an early stage of repair since its presence was required before the start of gap-filling repair synthesis (Shivji et al., 1994). The XPC protein is complexed tightly with hHR23B, one of the two human homologs of the yeast NER protein, RAD23 (Masutani et al., 1994). XPC-hHR23B protein complex is specifically involved in NER of DNA lesions on transcriptionally inactive sequences as well as the nontranscribed strand of active genes (Sugasawa et al., 1997).

All these purified proteins together can reconstitute DNA replication *in vitro*. By using isolated CTAG cell extract from an immortal, rapidly proliferating, SV40 large T antigen-transformed XP-V cell line which yields exacts which is highly active in the SV40 origin-based *in vitro* replication assay (Li and Kelly, 1984, 1985; Stillman and Gluzman, 1985), XP-V defect can be demonstrated.

I.7. current development to detect XP-V defect by *in vitro* assay system

Combination of an appropriate template carrying the SV40 origin of DNA replication with nucleotides, cell extract, and a single SV40 protein, large T antigen, yields completely resolved daughter molecules derived from a bi-directional, semi-conservative replication mechanism (Li and Kelly, 1985; Stillman and Gluzman, 1985). Using such a system with 254 nm UV-irradiated SV40-based DNA templates incubated in replication-proficient extracts made from XP-V cells compared to normal control, Svoboda et al. (1998) has provided the first clear demonstration of a replication defect on a T<>T-containing template using an XP-V cell extract, 10 to 26-fold deficiency in the bypass replication of T<>T in XP-V cell extracts was observed. This assay has already yielded novel mechanistic and quantitative information on the replication of T<>T in normal cell extract (Svoboda and Vos, 1995). It is possible to isolate the factor(s) which restore the ability of XP-V cell extracts to replicate a DNA template containing T<>T and it is also necessary to elucidate the bio-chemical processes operating in both normal and XP-V cells. In normal cells, the XP-V correcting factor may interact with UV-irradiated DNA to facilitate translesion synthesis past T<>T. The level of replication blockage by T<>T in normal human cells is relatively low. It is the abnormal interaction of T<>T with an unknown replication factor that results in deficient translesion bypass in XP-V cell extracts, and increased mutagenesis at A:T base pairs. Isolation of the XP-V correcting factor will lead to isolation of the XP-V gene, and an understanding of its role in neoplasmic transformation (Svoboda, 1996).

I.8. objective of present studies

It has been demonstrated that the genetic group XP-V of Xeroderma Pigmentosum (XP) which is an autosomally inherited genetic disorder characterized by extreme sensitivity to sunlight (Cleaver and Kraemer, 1989; Vos, 1995), show the clinical characteristics of the disease, is high incidence of skin cancer (Seguin et al., 1992; Boyer et al.,1990),but has normal or nearly normal rate of necleotide excision repair (Cleaver and Thomas, 1969; Burk et al., 1971; Cleaver, 1972; Wang et al., 1993), but is significantly more sensitive to the mutagenic effect of UV light (Wang et al., 1991).

There is evidence suggesting that XP-V cells carry a defect in replication of UV-induced damaged DNA, leading to mutations in genes, e.g. proto-oncogenes and tumor supressor genes, of exposed skin cells (Lehman et al.,1975; Dumaz et al.,1993; Misra and Vos,1993; Wang et al., 1993). In normal cells, the XP-V correcting factor may interact with UV-irradiated DNA to facilitate translesion synthesis past T<>T (as well as other photoproducts). The evel of replication blockage by T<>T in normal human cells is relatively low (Svoboda et al., 1998). *In vitro* studies with simian virus 40(SV40)-based minireplicons carrying randomly distributed UV induced damage have indicated that translesion bypass of the cyclonbutane pyrimidine dimers could be detected in normal human cell extracts (Li and Kelly, 1985; Carty et al., 1993). It is the abnormal interaction of T<>T with an unknown replication factor that results in deficient translesion bypass in XP-V cell extracts, and increased mutagenesis at A:T base pairs.

The objective of my project is to isolate the Xeroderma Pigmentosum-Variant correcting factor from normal human embryonic kidney 293 cells and also to develop a purification scheme over successive chromatography columns.

To achieve these aims, I have grown large quantity of human 293 cells in suspension conditions, and fractionate cell extracts which start from phosphocellulose column to detect complementing factor(s) present in normal human cells by *in vitro* DNA replication assay.

MATERIALS & METHODS

II.MATERIALS AND METHODS

II.1. MATERIALS

II.1.1. Chemical Reagents/Enzymes/Culture Media and Plastic Culture flasks

II.1.1.1 Chemical Reagents/drugs

Reagent/Drugs	M.W.	Cat #	Company
Pepstatin A	685.9	P-4265	Sigma
Aprotinin	4.0 TIU/mg	A-1153	Sigma
Leupeptin	475.6	L-2884	Sigma
Na butyrate	110.1	B-5887	Sigma
PMSF	174.2	195381	ICN
Myo-Inositol	180.16	102052	ICN
Boric acid	61.83	195074	ICN
Kanamycin sulfate	743mcg/mg	11815-024	Gibco
Benzamidine-HCl	156.6	B-6506	Sigma
DTT	154.2	D-0632	Sigma
calf serum	500 ml	16170-078	Gibco
fetal bovine serum	500 ml	26140-079	Gibco
fetal bovine serum	500 ml	16140-071	Gibco
L-glutamine (200 mM)	100 X	25030-081	Gibco
sodium pyruvate	25 g	11840-030	Gibco
penicillin “G”	356.4 (100,000,000 U)	P-3032	Sigma
streptomycin sulfate	787 U/mg	S-9137	Sigma
Trypsin-EDTA	500 ml	25300-062	Gibco
gel filtration standard	18 mg/vial	151-1901	Bio-Rad
NP-40	50 ml	155942	ICN

ampicillin	5 g	A-9518	Sigma
albumin, bovine		A-4503	Sigma
Trypan blue stain	0.4 %	15250-061	Gibco
rATP,rUTP,rCTP,rGTP	100 mM/each	27-2035-01	PMB
dATP	579.2	27-2050-01	PMB
dGTP	595.1	27-2070-01	PMB
dTTP	570.1	27-2080-01	PMB
ethidium bromide	100 mg/tablet	806808	ICN
Protein Assay Dye Reagent Concentrate		500-0006	Bio-Rad
Scintillation liquid solution: Formula-989		6NE9899	PIC

II.1.1.2. Enzymes

Enzyme	Conc.	total amount	Cat #	Company
MfeI	10,000 U/ml	500 units	589S	Biolabs
DpnI	20,000 U/ml	1,000 units	176S	Biolabs
MboI	10 U/ml	200 units	15248-016	Gibco
dam methylase	8,000 U/ml	500 units	222S	Biolabs
SAM	32 mM	100 µl	007-SAM	Biolabs
T_4 DNA polymerase	1 U/ µl	500 units	1004794	BMG
T_4 DNA ligase	5 U/ µl	500 units	716359	BMG
T_4 polynucleotide kinase	10,000 U/ml	2,500 units	201-L	NEB
T_7 sequenase		200 units	70775	ALS
proteinase K		100 mg	745723	BMG
creatine kinase		20 mg	126969	BMG
creatine phosphate		MW 327.2	127574	BMG
SV40 large T antigen	1.67 or 2 mg/ml	100 µg	5800-02	MBR

N.B.

1. ALS: Amersham Life Sciences, Inc. (Canada or Cleaveland, Ohio, USA)
2. Biolabs or NEB: New England Biolabs Ltd. (Mississauga, Ontario, Canada)
3. BIO-RAD: Bio-Rad Laboratories, Inc. (Richmond, CA, USA)
4. BMG: Boehringer Mannheim GmbH (W.-Germany)
5. Gibco: Gibco BRL Life Technologies (Burlington, Ontario, Canada)
6. ICN: ICN Biomedicals Inc. (Aurora, Ohio, USA)
7. MBR: Molecular Biology Resources Inc. (Milwaukee, USA)
8. PIC: Packard instrument company, Inc.(meriden, U.S.A)
9. PMB: Pharmacia Biotech (Baie d`Urfé, Quebec, Canada)
10. Sigma: Sigma Chemical Corporation (St.Louis, MO., USA)

II.1.1.3. Cell lines/Cell Culture Media and Supplements/Culture flasks

II.1.1.3.1 Cell Lines

293 cells were obtained from Dr. Bruce Stillman (Cold spring harbor laboratory, Cold spring harbor, NewYork 11274, USA), were human normal embryonic kidney cells transformed with fragments of adenovirus types 5 (ad5) DNA.

CTAG or XP4BE-SV cells were obtained from Dr. William Kaufmann (Lineberger comprehensive cancer center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-295), were fibroblast of XP-V male patient transformed by TAg.

II.1.2.3.2. Culture Media and supplements

Joklik's modification of miminal essential medium (J.M.MEM) (ICN#10-323-24) contains sodium bicarbonate, L-glutamine 294 mg/l, penicillin 75,000 IU, streptomycin 50 mg/l, supplemented with 5 % calf serum for 293 cells.

Minimum Essential Medium (MEM) (Gibco #61100-012), 47.6 g/pkg, with earle's salts,L-glutamine,without sodium bicarbonate, supplemented with 10% fetal bovine serum (Hyclone,heat-inactivated), L-glutamine (4 mM),penicillin (115 units/ml) and streptomycin (115 units/ml) for CTAG cells.

II.1.1.3.3. Culture Flasks

culture flasks were purchased from Nunc Inter Med (GIBCO-BRL, burlington, Ontario,Canada), Falcon (Becton Dickinson and Company, Franklin lakes, NJ.,USA). The 1 ml, 2 ml, 5 ml, 10 ml, 25 ml and 50 ml disposable sterile pipettes were purchased from Costar Corporation (Cambridge, MA, USA).

II.1.2. Stock Solutions/Buffers

II.1.2.1. Stock Solution

creatine kinase: 2.5 mg/ml in 20 mM HEPES, pH 7.8
sodium phosphate: 250 mM, pH 7.5
proteinase K: 20 mg/ml
6 x gel loading solution: 0.25% bromophenol blue,0.25% xylene cyanol FF, 40% glycerol, 0.6% SDS
fixing solution: 10 % acetic acid plus 10 % methanol
Trypsin-EDTA: 0.05 % Trypsin, 0.53 mM EDTA-4Na

II.1.2.2. Buffers

II.1.2.2.1. NEB buffer #4: 50mM potassium acetate,20mM Tris acetate,10mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C

II.1.2.2.2. buffers for cell extracts:

II.1.2.2.2.1. 1 x PBS: dilute 10 X phosphate saline buffer with deionized water

II.1.2.2.2.2. Hypotonic buffer:

20 mM Hepes, pH 8.0, 5 mM KCl, 1.5mM $MgCl_2$, 1 mM dithiothreitol, 5 mM sodium butyrate (for 293 cell extract)

II.1.2.2.2.3. Isotonic buffer: hypotonic buffer + 250 mM sucrose

II.1.2.2.3. buffer for Q-sepharose column:

buffer B+ 50 mM or 1 M NaCl: 25 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA, 10 % glycerol, 0.1% NP-40, 0.1 mM PMSF, 1 mM DTT, 5 mM sodium metabosulfite, 50 mM or 1M NaCl

II.1.2.2.4. buffers for cellulose phosphate (P-11) column:

II.1.2.2.4.1.buffer H + 15mM or 100mM KCl (dialyze,sephacryl HR-200 column buffer or conductance buffer): 30 mM Hepes, pH 7.8, 0.25 % inositol, 0.25 mM EDTA, pH 8.0, 1 mM DTT, 0.1 % NP-40, 15 mM or 100 mM KCl

II.1.2.3.4.2. buffer F + 100 mM KCl (equilibrate buffer): 30 mM Hepes, pH 7.8, 0.25% inositol, 1 mM DTT

II.1.2.3.4.3. buffer P + 500 mM or 2 M KCL (elution buffer):

80 mM potassium phosphate, pH 7.2, 0.25 mM EDTA, 0.25 % inositol, 1 mM DTT, 500 mM or 2 M KCl

II.1.2.5. buffers for phenyl sepharose column:

II.1.2.5.1. starting buffer: buffer H + 1 M KCl or 1 M ammonium sulfate + 50 mM sodium phosphate

II.1.2.5.2. elution buffer: buffer H + 50 mM, 100 mM, 200 mM, 500 mM, 600 mM or 50 mM sodium phosphate

II.1.2.6. 1 x TBE buffer for 0.8 % agarose gel: 0.09 M Tris-Bonate, 0.002 M EDTA (pH 8.0), 0.09 M boric acid

II.1.2.7. 10 x annealing buffer:

200 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 500 mM NaCl

II.1.2.8. 10 x synthesis buffer for second strand synthesis:

5 mM dNTPs,10 mMATP,100 mM Tris-HCl pH 7.4, 50mM $MgCl_2$,20 mM DTT

II.1.2.9. 20 x dNTP: 2 mM dATP, dGTP, dTTP, 400 uM dCTP

II.1.2.10. 5 x T_7 sequenase reaction buffer:

200 mM Tris-HCl, pH 7.5, 100mM $MgCl_2$, 250 mM NaCl

II.1.2.11. LB-agar medium (1 L):

bacto-tryptone 10 g, bacto-yeast extract 5 g, NaCl 10 g, bacto-agar 17 g

II.1.2.12. 2 x YT medium (1L):

Bactoo-Tryptone16 g, bacto-yeast 10 g, NaCl 5 g

II.1.3. Isotopes

Thymindine 5'-Triphosphate [methyl-^{3}H]- ^{3}H-TTP (Cat. # NET221A, 400 µl, 80.9ci/mmol) was purchased from Dupont, NEN (Boston, MA, USA)

[α-^{32}P]dCTP was purchased from Dupont, NEN (Boston, MA, USA) (Cat. # 513H05148, 25 or 50 µl, 3,000ci/mmol, 10mci/ml) easytides™

II.1.4. columns/resins

columns/resins	Cat #	company
BND cellulose column		Sigma
Qiagen tip-100 column	10043	Qiagen Inc., Chatsworth, USA
Q-Sepharose fast flow	17-0510-10	Pharmacia Biotech
Cellulose Phosphate P-11	4071010	Whatman International Ltd. Springfield Mill, Maidstone, Kent, England
HiTrap HIC Test Kit ----- Phenyl Sepharose Fast Flow (low sub)	17-1349-01	Pharmacia Biotech
HiPrep Sephacyl S-200 High Resolution 16/60	17-1166-01	Pharmacia Biotech
sepharose CL-4B	17-0150-01	pharmacia biotech AB, Uppsala Sweden, Pharmacia Biotech

Q Sepharose Fast Flow	17-0510-10	pharmacia biotech AB, Uppsala Sweden, Pharmacia Biotech
Protein A Sepharose CL-B	17-0780-01	pharmacia Biotech AB (Uppsala Sweden)

II.1.5. special equipments and products

KODAK Scientific Imaging Film (BioMax film) Cat# 8701302 (8 x 10 in., 20.3 x 25.4cm) interscience (eastman Kodak company, rochester, New York, USA)

Spectra molecularporus membrane tubing MWCO: 12-14,000, 16 mm, Cat# 132 678 [Spectrum Laboratories, Inc. (Companies), Laguna Hills, Canada]

Phosphorimager GS-525 multi-analysist system, Bio-Rad laboratories, Hercules, Canada

1217 Rockbeta liquid scintillation counter, LKB, Wallac

Sorvall RC3B centrifuge, mandel, Scientific Co.Ltd, St.Laurent, Quebec, Canada

Sorvall OTD-COMBI ultracentrifuge, Du Pont Company, Wilmington, Delaware, USA

II.2. METHODS

II.2.1. Cell cultures

II.2.1.1. 293 cells were obtained from Dr.Bruce Stillman (Cold spring harbor laboratory, Cold spring harbor, NewYork 11274, USA), were human normal embryonic kidney cells transformed with fragments of adenovirus types 5 (ad5) DNA (Graham et al., 1977). Cells were grown in Joklik's modification of miminal essential medium (J.M.MEM) (ICN#10-323-24) which contains sodium bicarbonate, L-glutamine 294 mg/L, penicillin 75,000 IU, streptomycin 50 mg/l supplemented with 5 % calf serum (heat-inactivated, Hyclone) in suspension conditions in spinner flasks at density of 0.2 x 10^6 cells/ml at 37 °C. Doubling time is around 24 h. Every other day, cells were counted with a hemacytometer, and after gentle mixing, cells were tranferred into the next larger spinner flasks, with the addition of an appropriate amount of fresh JMMEM medium to maintain the same density. When the cells reach 0.5-0.6 x 10^6 cells/ml/6l flask, cells were harvested, and extracts prepared, holding some cells in reserve to maintain the culture for the next step.

II.2.1.2. CTAG cells (CRL 1162 from ATCC; Kaufman,1995), fibroblasts from an XP-V male patient and transformed by SV-40 large T antigen, were obtained from Dr. William Kaufmann (Lineberger comprehensive cancer center, University of North Carolina at Chapel Hill).CTAG cells were grown in MEM medium (Gibco #61100-012) supplemented with 10 % fetal bovine serum (Hyclone, heat-inactivated), L-glutamine (4 mM), penicillin (115 units/ml) and streptomycin (115units/ml) in tissue culture flasks at density of 0.5 x10^6 cells/ml (These are not suspension cells. They were grown in sub-confluent, attached cultures) at 37 °C in atmosphere with 5 % CO_2.Every other day, the medium was changed, and when CTAG cells grew to reach 90-100 % confluence (in 175 cm^3 culture flask), the culture was trypsinized, and split 5:1. The culture was expanded until there were 20 x 250 cm^3 flasks. When these flasks reached ca. 80 % confluency, cells were harvested by trypsinization, followed by preparation of cell extract (see below).

II.2.2. Preparation of cell extracts

II.2.2.1. 293 cell extracts (293-S100):

The cytoplasmic extracts of human 293 cells (293-S100) were prepared as described (Stillman and Gluzman, 1985; Li and Kelly, 1985). Only used cells that were doubling in less than or equal to 24 hours. Cells with longer doubling times were discarded and a new culture started. Cells from 6-15 liter culture were harvested during the mid-log phase, at a density of 0.5-0.6 x10^6 per ml (the cell density was measured to estimate the total number of cells used for the extract preparation) in 6 x 1 liter bottles (Sorvall, prechilled on ice) by centrifugation at 2,500 rpm for 10min at 4 °C. Kept bottles on ice,the media was removed, and the cells were pooled into two 250 ml bottles (Sorvall) after gentle pipetting and the remaining bottles rinsed serially with about 25 ml of ice-cold 1x phosphate-buffered saline (PBS), and added to the pooled cells. Cells were collected by centrifugation at 2,500 rpm for 3 min at 4 °C, resuspened by gentle pipetting with 5 ml of prechilled PBS per liter bottle (20 ml PBS/0.5-0.6 x 10^6 cells/4 liter cell cultures), then transferred cells to 50 ml Falcon tubes on ice. Cells again were collected by centrifugation at 2,500 rpm for 3 min at 4 °C, the supernatant was carefully removed, and the volume of packed cells in each tube noted down in order to determine the volume of hypotonic buffer to be used. Added fresh, ice-cold Hypotonic buffer (20 ml Hypotonic buffer/7-8 ml cell pellets), and resuspended cells by gentle pipetting. After pelleting cells once again, the supernated was aspirated,and cells were resuspended in 1 ml Hypotonic buffer for each ml of packed cell volume.Cells were allowed to swell for 10 min on ice, and at the same time, protease inhibitors were added (100 mM PMSF, 1 M Benzamidine-HCl, 0.7 mg/ml Pepstatin A, 1 mg/ml Aprotinin, 0.5 mg/ml Leupeptin of 1/500 volume and 1 M Na bisulfite of 1/200 volume of cell suspension). The cells were gently mixed until homogeneous and then transferred to an appropriate sized glass Dounce homogenizer on ice. The cells were examined under a phase contrast microscope by taking 10 µl mixing with the same volume of Trypan blue stain (0.4 %, Gibco). Then, cells were disrupted by applying 20-25 strokes (up & down for 40-50 passages) with a "B" pestle, and stained

and reexamined under the microscope. If less than 85 % of the cells were broken, several more strokes with the B pestle were applied. The mixture was then transferred to two (or four) 30ml Corex tubes, allowed to incubate on ice for 30min and then centrifuged at 10,000 rpm for 10 min at 4 °C with Sorvall-34 rotor. The supernatant was carefully removed and pooled into one (or two) 50 ml Falcon tube to measure the volume, added 5 M NaCl to 0.1 M final concentration and mixed gently.

The supernatant was carefully transferred to ultracentrifuge tubes and centrifuged in pre-chilled T865.1 rotor for 60min at 100,000 g (37,000rpm) at 4 °C. The extract (supernatant) was then dripped slowly into liquid N_2. The N_2 was decanted and the frozen cell droplets were stored at -70 °C. Prior to use in a replication assay, 50 µl of the frozen droplets was thawed gently and centrifuged at 4 °C for 2-3 min in a microfuge, be careful not to take any precipitated material into the reaction. The protein concentration of the extract was typically between 5 and 10.5 mg/ml as determined by Bio-Rad Laboratories assay with bovine serum albumin as the standard (Bradford, 1976). The 293 cell extracts may be frozen and thawed multiple (>3) times without significant loss of replication activity.

II.2.2.2. CTAG cell extracts:

Cytoplasmic extracts of CTAG cell were prepared as above with some modifications (Stillman & Gluzman, 1985). CTAG cells from 20 x 250 mm^3 culture flasks were harvested by trypsinization and centrifugation when they reached ca.80% confluency. Cells were then washed and resuspended with an appropriate amount of isotonic buffer (0.25 ml Iso/1 x10^6 cells/ml culture) twice, then added hypotonic buffer (1 ml/1 x 10^8 cells/ml culture) without adding protease inhibitors to yield a cell density of about 1 x10^8 cells per ml. Swell on ice for 15 min, then disrupted the cells by about 15 strokes with "B" pestle in Dounce homogenizer on ice, incubated for 30 min, then transferred to eppendorf, centrifuged twice at 12,000 rpm for 10 min at 4 °C, aliquoted 50 µl then froze down, each time used fresh one to do the replication assay.

II.2.3. Preparation of DNA templates

The T<>T containing and TT control template were prepared by annealing the original undamaged or T<>T containing 20 nucleotide oligomer to a circular, single-stranded DNA derived from pBluescript II KS(-) (Svoboda and Vos, 1998).

First, 1000 pmol of the T<>T containing 20 mer oligonucleotide (Svoboda and Vos,1995) was phosphorylated at the 5' end by treatment with a 100-fold molar excess of ATP and 100 Units of T_4 polynucleotide kinase in 10 µl of the buffer supplied by the manufacturer (NEB) at 37 °C for 60 min, followed by treatment 65 °C for 10 min. Then, single stranded M3- DNA (Svoboda and Vos, 1998) was prepared according to the protocol as described (Maniatis et al.,1985). Phosphorylated, T<>T 20 mer was annealed with single-stranded DNA by slowly cooling from 75 °C to 35°C in annealing buffer (20 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 50 mM NaCl). A standard reaction contained 10 pmol ssDNA (ca. 11 µg), 20 pmol phosphorylated 20 mer at a final annealing volume of 500 µl. After cooling on ice, T_4 DNA polymerase (50U; 1U/µl), T_4 DNA ligase (200 U; 5 U/µl), 10 µl 50% glycerol, and 50 µl 10x synthesis buffer (5 mM dNTPs, 10 mM ATP, 100 mM Tris-HCl pH 7.4, 50 mM $MgCl_2$, 20 mM DTT) were added and vortexed well. Typically, 10 such mixtures were prepared, and allowed to remain on ice for 5 min., followed by 5 min. at room temperature and 90 min. at 37 °C. After heat treatment at 65 °C for 10 min, reactions were checked on an ethidium containing agarose gel (0.8 %) to confirm the production of dsDNA. DNA was purified using Qiagen columns (2 x Tip 100, according to the theoretical yield of ca. 220 µg). Columns were equilibrated with QBT buffer (manufacturer by Qiagen; Sambrook et al.,1989), DNA solution was mixed 1:1with QBT buffer, and applied to the columns. Columns were washed with buffer QC, eluted with buffer QF, and the DNA precipitated with isopropanol according to procedures outlined in the Qiagen manual (manufacturer by Qiagen; Sambrook et al., 1989).

The DNA pellet was then dissolved in ca. 500-700 µl deionized H_2O, and the concentration determined by measurement of the absorbance at 260 nm. Then, the newly

synthesized strand was methylated by adding 100 U dam methylase (Biolabs, 8 U/ μl), together with 2.5 μl of 400 x SAM (32 mM), 100 μl of 10 x dam methylase buffer as supplied by the manufacturer, and deionized water to a final volume of 1 ml. The mixture was incubated at 37 °C for 2.5 h, followed by digestion of an aliquot with 0.75 U DpnI (under the same conditions as in the replication assay) to check for complete methylation at GATC sites. After heat inactivation of the enzyme at 65 °C for 10 min., exonuclease III (3 U/μg DNA) was added to digest any unligated second strands. DNA was isolated from the mixture by application of Qiagen columns as above.

Finally, dsDNA was isolated by application to a well-washed (to remove UV-absorbing material) BND cellulose column (Sigma) with 20 mM Tris-HCl, 1 M NaCl, pH 7.4, 5 mM EDTA. Under these conditions, DNA that contains single stranded regions binds tightly to the column resin, while dsDNA washes off. Yield is typically between 20 and 50 μg.

II.2.4. Internal standard preparation

High specific activity, [^{3}H]-labeled DNA was prepared by annealing 0.5 ul of 2.5 pmol/μl 20 mer and 1.25 μl of 1 μg/μl ssDNA in 2 μl of 5 x sequenase buffer in a total volume of 10 μl. To label the DNA, 1 μl of 1 M DTT, 2 μl of 2 mM dA,C,GTP, 50 μl of [3H]-TTP (80.9 Ci/mmol), 10.9 μl 5 x sequenase buffer, 2 μl sequenase (26 U), and deionized water to a total volume of 100 μl were added. The mixture was held on ice (5 min.) and at room temperature (5 min.), followed by incubation at 37 °C for 90 min. Labeled DNA was isolated on a 1 ml Sepharose CL-4B column. An aliquot of 5 μl was counted for [^{3}H] activity, and if the cpm value was near 100,000, stored at -20 °C for experimental use.

II.2.5. *In vitro* DNA replication assay

The *in vitro* DNA reactions were performed as described previously (Li and Kelly, 1985) with some modifications. The standard 12.5 μl reaction mixture contained 30 mM HEPES (pH 7.8), 7 mM $MgCl_2$, 200 μM rCTP/rGTP/rUTP, 4 mM rATP; 100 μM dATP/dGTP/dTTP, 20 μM dCTP; 40 mM creatine phosphate; 100 μg creatine kinase; 15mM sodium phosphate; 1.25 μCi α-^{32}P-dCTP (3,000 Ci/mmol); 12.5 ng DNA template (TT or T<>T); 3.25 μl cell extract; 3.25 μl of column fraction or 50 % glycerol; 0.5 μg SV40 large T Antigen. Mixtures were incubated at 37°C for 90 min., and quenched by adding 0.5μl 20% SDS, 2 μl 20 mg/ml proteinase K,and 5μl [^{3}H]-DNA internal standard. Quenched reactions were then incubated at 50 °C for at least 60 min. This was followed by isolation of DNA on a 1 ml Sepharose CL-4B column using NEB restriction digestion buffer #4. DNA was eluted in the void volume, and small molecules including digested protein, and the proteinase K were retained. After washing with 300 μl buffer #4, the 150 μl containing ^{32}P-labeled DNA replication products, and ^{3}H-labeled internal standard DNA were collected.

After isolation of the products, 20 μl of each sample were digestion with mixtures of restriction enzymes appropriate to the analysis.For the "plus MfeI" enzyme group,0.5 U MfeI was added and incubated at 37°C for 1 h,followed by inactivation at 65°C for 15 min. For the "minus MfeI" group 50 % glycerol was substituted for enzyme to give a mock digestion. Then, all samples were treated with 0.5 U MboI (an enzyme that only digests DNA which is unmethylated on both strands), 0.75 U DpnI (an enzyme that only digests DNA which is methylated on both strands), 3 μl 2M NaCl, 1 μl 10x NEB buffer #4, and deionized water to a final volume of 30 μl, followed by incubation at 37°C for 1 h. Five microliters 6 x gel loading solution was added, and 5 μl aliquots were counted in a scintillation counter to determine ^{32}P and ^{3}H. After, normalizing the samples for ^{32}P incorporation and ^{3}H recovery, samples were electrophoresed on a 0.8 % agarose gel with ethidium bromide, 2-3 h at 200 V. After fixing gels in 10% acetic acid, 10% methanol, gels were dried at 80°C for 1-2 h.Autoradiographs of dried gels were prepared using either KODAK Scientific Imaging Film, or Bio-Rad GS-525 phosphorimager system. Covalently

closed circular DNA after MfeI digestion indicates that bypass replication through the T<>T was present (Figures 9, 10).

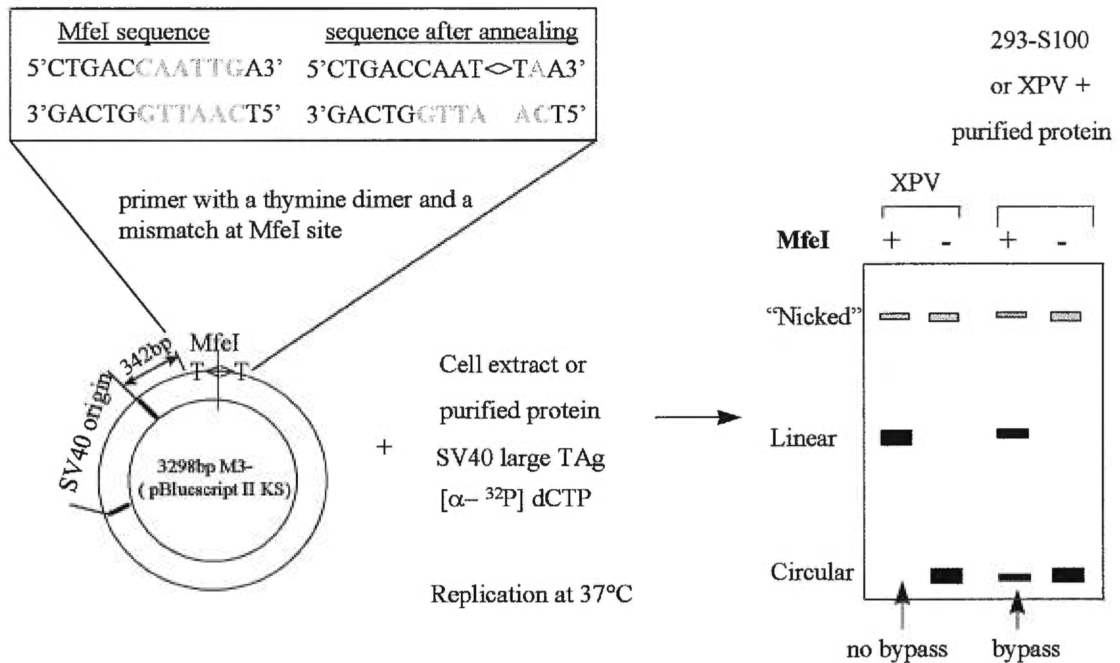

Figure 9. Interpretation of thymine dimer template preparation and bypass replication by *in vitro* DNA replication assay. Single thymine dimer at MfeI site was 342 bp away from SV40 origin in plasmid M3-. After replication with the addition of cell extract or purified protein, and restriction digestion, agarose gel electrophoresis shows that addition of active fractions restores bypass replication to XPV cell extract.

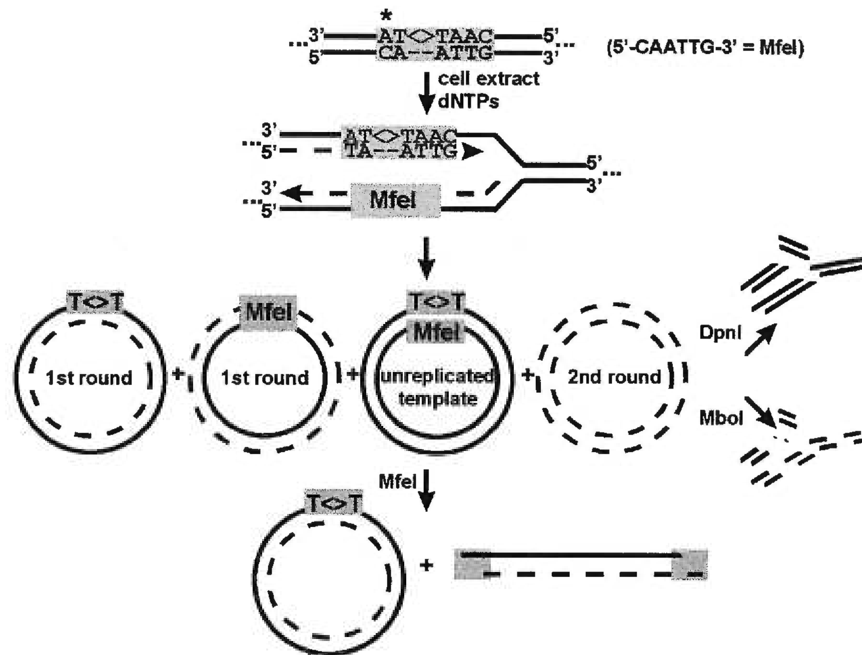

Figure 10. Interpretation of enzyme digestion, details in text.

DNA template preparation results in a synthetic, double-stranded DNA molecule with a unique MfeI site in the undamaged strand opposite the T<>T (or TT in the control template). After replication in human cell extract, only daughter molecules synthesized from the undamaged strand are sensitive to MfeI digestion. Therefore, digestion with MfeI selectively eliminates from the Form I band on the autoradiogram, the contribution by replication of the undamaged template strand. Another source of backround Form I is generated from non-specific incorporation of short patches of nucleotides (without TAg). This is removed by digestion with the methylation-specific DpnI endonuclease yielding small fragments. Second-round daughter molecules are digested with MboI, which only digests DNA which is unmethylated on both strands. Treatment with MfeI linearizes daughter Form I molecules synthesized from the undamaged strand, leaving daughter molecules synthesized from the T<>T containing strand as the sole, label containing Form I molecules. Replication of the T<>T containing template strand is revealed by the presence of a Form I gel band following the MfeI/DpnI/MboI restriction enzyme digestions (Svoboda et al., 1998) especially after MfeI digestion.

II.2.6. Quantitation of bypass replication

Autoradiograms created using the Bio-Rad GS-525 phosphorimager system were analysed using the software included with the instrument. The closed circular (Form I) DNA bands from replication of each sample using T<>T containing template after MfeI digestion were compared after normalization with respect to basal replication (^{32}P incorporation) using TT control template. There are 2 ways this could be accomplished. 1. Using the ^{3}H-labeled internal standard to adjust the quantity of each sampled loaded onto the gel, the each lane in the gel image is directly representative of DNA synthesis in each reaction.Consider the conditions used in a hypothetical lane #1 where we have loaded DNA from a reaction using XPV cell extract plus fraction #1. We have total ^{32}P incorporation with control template (determined from scintillation counting of samples and normalizing to ^{3}H) that is, for example, 1.5 times that than the level of ^{32}P incorporation with control template when XPV cell extract is added to fraction #2. Then the Form I

DNA band on the gel needs to be normalized to reflect differences in the basal (TT control template) level of replication (^{32}P incorporation). In this way, any difference in T<>T bypass due simply to the level of replication using cell extract and any particular column fraction can be eliminated.

Alternatively, in the second method, which we chose to employ, the same type of normalization was performed prior to running the gel by adjusting the amount of sample loaded in each lane so that Form I bands could be compared directly. In this way, in any one experiment, the level of T<>T bypass due to a column fraction could be evaluated by visual inspection of the gel image, and a simple determination of the number of counts associated with the Form I band.

II.2.7. Packing and running of phosphocellulose (P-11) column

Phosphocellulose resin (Whatman P-11, 3.3 g) was placed into 300 ml beaker, and added 0.5 M NaOH 82.5 ml (25 x). The suspension was mixed well with a glass rod, and allowed to settle. After removing the supernatant gently, the resin was washed 3 x with 200 ml of deionized water, checking pH of the filtered supernatant, until the pH was less than or equal to 11. After settling, the upper layer was removed gently, and the same volume of 0.5N HCl was added,and mixed well. Again, after settling,the resin was washed with deionized water util the pH of the supernatant was greater than or equal to 3.

To 32 ml of the prepared resin was added with 8 ml of buffer F + 100 mM KCl according (settled volume plus 20 %).A 5 ml column was packed by,mixing the resin well, and pouring about 10 ml of the slurry into the column all at once. The resin was then washed with at least 10 x bed volume with buffer F + 100 mM KCl. Adjusted the baseline of the UV detector (280 nm) with the flow rate 0.1 ml/min until stable. Alternatively, for a larger amount of sample, a 65 ml (12.2 cm long and 2.6 cm diameter) column was packed, and equilibrated at a flow rate of 0.5 ml/min. Protein was loaded at ca.5-8 mg/ml column.

Cell extract (293 cells; S100 extract, see above) was thawed at 10 °C, and after centrifugation at 1,000 rpm, 10 °C for 3 min, added PMSF into the supernatant to a final concentration of 1.1 mM. Then, the sample was centrifuged at 4°C, 8,000 rpm for 30 min, followed by adjusting the conductivity of the supernatant to be the same as buffer H + 100 mM KCl, by adding 3 M KCl dropwise and monitoring the conductivity with an Orion model conductivity/Salinity/temperature meter. The clarified and salt-adjusted cell extract was applied to the column through the sample reservoir by gravity,and the column washed with buffer F + 100 mM KCl at a flow rate 0.1 ml/min. Fractions of 1 ml were collected, and after UV detection of the first peak (CFI), buffer was changed to buffer P + 500 mM KCl. A second peak was eluted (CFII). The remaining protein was eluted by buffer P + 2 M KCl.

Protein concentration of the column fraction was determined by Bio-Rad dye reagent assay. Fifty microliters of each fraction was mixed with 12.5 µl Bio-Rad concentrated dye reagent in the wells of a 96 well micro-titer plate. Fractions producing a strong color change were pooled yielding CFI and CFII. The pooled fractions were dialyzed against a volume of at least 100 x buffer H + 15 mM KCl at 4 °C overnight, with one buffer change. Samples were then aliquotted, frozen in liquid N_2, and stored at -80°C.

II.2.8. Running of Sephacryl high resolution (HR) 200 column

HiPrep16/60 Sephacryl S-200 column (Pharmacia), containing 120 ml resin was equilibrated at 4 °C with 2 bed volumes of buffer H + 15 mM KCl at a flow rate of 0.5 ml/min. The column was calibrated for elution volume vs. molecular weight by loading a protein standard solution (300 µl/10.8 mg, Bio-Rad). The standard curve had 5 peaks: A + B, thyroglobulin, 670 kDa; C, gamma globulin, 158 kDa; D, ovalbumin, 44 kDa; E, myoglobin, 17 kDa, and vitamin B-12, 1.35 kDa. High salt fraction (CFII, see above) was thawed on ice, and after centrifugation at 4 °C, 8,000 rpm for 30 min, loaded onto the Sephacryl HR-200 column and eluted with a flow rate of 0.5 ml/min, collecting 1 ml

fractions. Protein containing fractions were determined with the dye assay as above, frozen in liquid N_2, and stored at –80°C.

II.2.9. Phenyl sepharose column

A 1 ml column of phenyl sepharose, low loading (HiTrap column, Pharmacia) was equilibrated with 5 x bed volume with high salt buffer (H + 1 M KCl) or 1 M ammonium sulfate + 50 mM sodium phosphate. The combined active fractions from the Sephacryl HR-200 column were applied to the column. The bound protein was eluted with low salt buffer (H + 50, 100, 200, 500 mM KCl) or 50 mM sodium phosphate buffer. Fractions of 1 ml were collected, and protein determined by the BioRad dye assay as above. Flow through and eluted protein fractions were combined, dialyzed against Buffer H + 15 mM KCl, frozen in liquid N_2, and stored at –80°C.

II.2.10. Packing and running of Q-sepharose column

A 5.5 ml column of Q-Sepharose fast flow (Pharmacia) was packed by mixing the resin well, and gently pouring into the column. The resin was equilibrated with 10 bed volumes of buffer B + 50 mM NaCl,and continued to wash until the UV detector baseline was stable with flow rate of 1 ml/min. CFII (46 mg), which was dialyzed against buffer B + 50 mM NaCl was applied to the column by gravity, and step eluted with buffer B containing 50 mM, 150 mM, 250 mM, 350 mM, and 1M NaCl. Protein in the collected tubes was determined by BioRad dye reagent assay as above, and pooled to yield fractions QFT, QA, QB, QC, QD. These fractions were dialyzed against 100 volumes of buffer H +15 mM KCl at 4 °C overnight. Fractions were aliquotted, frozen in liquid N_2, and stored at –80°C.

RESULTS

III. RESULTS

Standard protein purification procedures were followed to isolate the XP-V correcting factor from human 293 cell extract.

III.1. bypass activity in 293 cell extracts

293 cells were grown in spinner flasks in suspension conditions and collected at 0.5-0.6 x 10^6 cells/ml. Cells were disrupted by dounce homogenization to yield 2132.21 mg protein in a volume of 319.4 ml, as determined by Bio-Rad protein assay. Cell extracts from preparations made from several different cultures grown to different final volumes all demonstrated replication bypass activity, and could complement the XP-V defect on thymine dimer containing template (Figure 11). Only the replication on T<>T template was

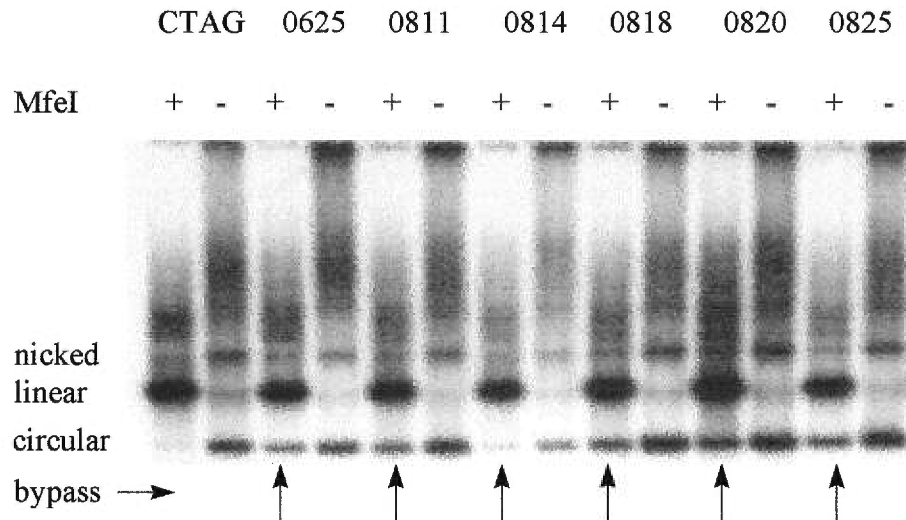

Figure 11. Defective and effective bypass replication of cyclobutane thymine dimer in XP-V and 293 cell-free extracts. Semisynthetic plasmid DNA molecules containing a single T<>T were incubated with normal human (293) and XP-V (CTAG) fibroblast cell extracts for 60 min. Labeled replication products were digested with MfeI digestion followed by agarose gel electrophoresis. 0625, 0811, 0814, 0818, 0820, 0825 were 293-S100 from different cultures grown to different final volumes of 293 cells.

shown in the figure. After replication with and without MfeI digestion, nicked, linear and circular DNA bands appeared on the agarose gel. In the negative control, CTAG (XP-V), the circular DNA band was not seen. There was no bypass replication through the T<>T, but 293-S100 from different volumes of 293 cells all had bypass activity. This demonstrated that 293 cell extracts can support DNA replication in the presence of T antigen and complemented XP-V defect on thymine dimer containing template.

III.2. Scheme of XP-V correcting factor purification procedure from the fractionation of 293 cell-free extracts 293-S100 (fig 12):

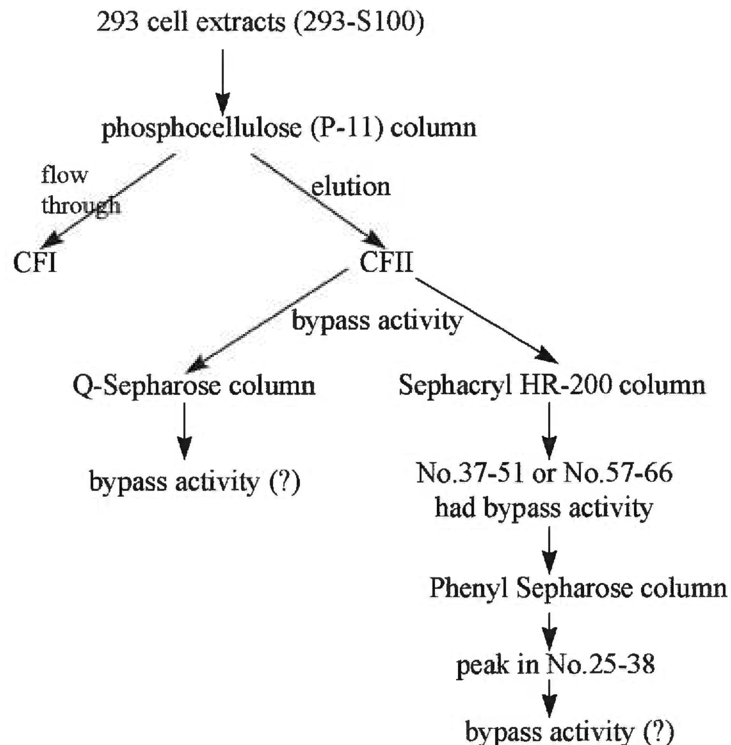

Figure 12. Scheme of XP-V correcting factor purification procedure. All purification work was performed at 4°C, and began with the phosphocellulose column.

A S100 extract from 293 cells was divided into multiple fractions by phosphocellulose chromatography and fractionated further to purify replication components. Prior to each step, replication activity was tested, and only the fraction with bypass activity was applied to the next column.

III.3. high salt fraction of phosphocellulose column from 293 cell extracts (293-S100) complements XP-V defect on thymine dimer containing template.

390 - 510 mg of 293-S100 were loaded onto the new-packed 65 ml bed volume of P-11 column (continuous use no more than one week) after low salt buffer equilibration.

Binding protein was eluted with different concentrations of salt at flow rate 0.5 ml/min. Two column fractions were obtained: flow through CFI and high salt elution CFII. Total amount of protein, protein recovery rate and replication unit for CFI & CFII were 1110.58 mg, 591.46 mg; 52.09 %, 27.74 % and 4.07-7.18%,33.57-45.16%, respectively (table 3). The bypass unit recovery of the bypass replication from T<>T template comparison with from the TT control was quantitated by the Bio-Rad phosphorimager system. Bypass replication unit recovery of CFI and CFII from both 293 and Hela normal cell extracts was less than 8 % and more than 20 % respectively, which was in CTAG cell extract only 3.47-6.35 % (data not shown). They determined that the less bypass replication unit recovery, the less bypass replication. These results were in agreement with those obtained from bypass replication in CTAG cell extract and CFI fraction (figure 13).

	Conc. (ug/ul)	Volume (ml)	Total amount (mg)	Protein recovery (%)	Replication unit recovery (%)
293 cell extract	7.00(5.00-10.24)	319.4	2132.21	100	18.68-18.99
CFI of P-11	2.97(1.46-5.80)	376	1110.58	52.09	4.07-7.18
CFII of P-11	1.85(1.60-3.04)	252.9	591.46	27.74	33.57-45.16
Hela cell extract	8.26	3	24.78	100	31.8
CFI of P-11	2.65	1.6	4.24	17.11	2.5
CFII of P-11	1.75	1.75	3.06	12.35	20.6

Table 3. comparison of the basic parameters of CFI, CFII of P-11 column fraction between 293 (n>10) and Hela (n=1) cell extracts. Protein concentration was determined by Bio-Rad assay using bovine serum albumin as the standard. Protein recovery was percentage of the amount of CFI, CFII divided by the amount of 293 or Hela cell extracts,

respectively. Replication unit recovery was estimated as the percentage indication of bypass replication by quantitation of each band of circular DNA on an agarose gel, which was compared from T<>T template replication to from TT control template replication measured by phosphoimager.

DNA replication of the SV40-based double-stranded DNA template carrying a single T<>T (or TT) incubated in phosphacellulose fraction CFI and CFII was analyzed by agarose gel electrophoresis after MfeI/MboI/DpnI digesion and normalization of ^{32}P and ^{3}H account (see figure 13). Replication of the T<>T containing template strand is revealed by the presence of a Form I gel band. After replication and enzyme digestion, nicked, linear and circular DNA (Form I) bands were obtained on 0.8 % agarose gel (only the results of replication on T<>T template were shown in the figure). Circular DNA band was not observed on the gel of the negative control (XP-V cell extract); but was seen from the replication of positive control 293-S100. After addition of phosphocellulose column fraction CFI and CFII to the CTAG extract, bypass activity was observed only in the high salt fraction, demonstrating bypass replication. The same results were obtained after centriprep-10 in 12.5 μl reaction solution with different amounts of CFI and CFII (0.1625 μl, 0.25 μl, 0.325 μl) (figure 14). This figure illustrated that only the high salt fraction

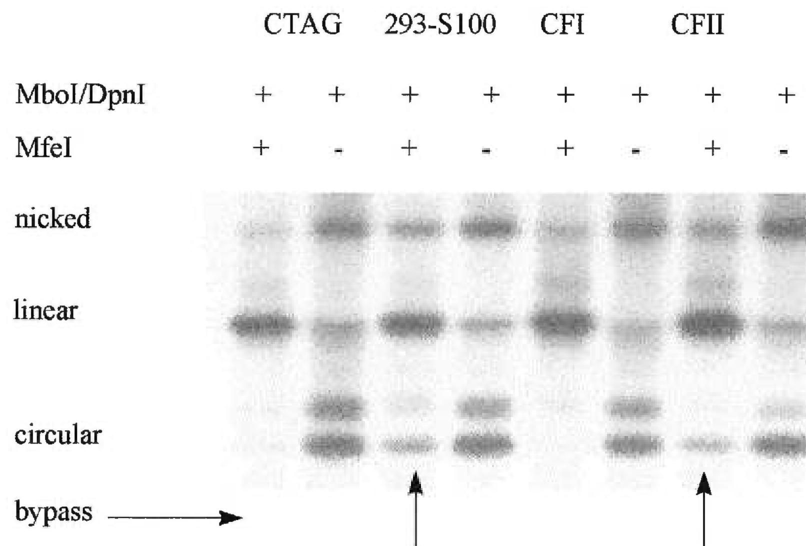

Figure 13. The high salt fraction of phosphocellulose column from 293 cell extracts complements XP-V defect on thymine dimer template with MfeI, MboI and DpnI digestion.

eluted from the phosphocellulose column had bypass activity, and can thus complement XP-V defect on thymine dimer containing template.

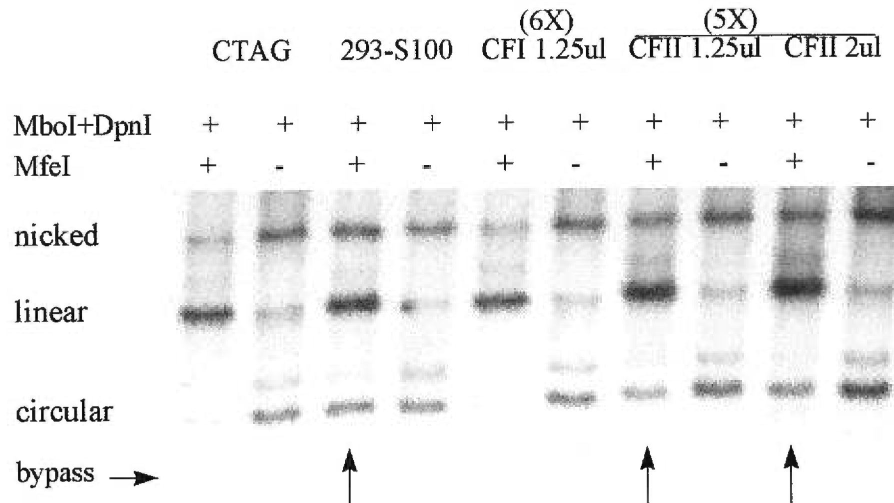

Figure 14. different amounts of high salt fraction of phosphocellulose column from 293 cell extracts complement XP-V defect on thymine dimer template.

III.4. Sephacryl HR-200 column fraction showed bypass activity, but did not separate well.

The working conditions and functioning of the pre-packed Sephacryl HR-200 column from Pharmacia were tested by loading 300 μl of protein standard (500 μl/18 mg, Bio-Rad Laboratory) onto the Sephacryl HR-200 column. The protein standard curve had the expected 5 peaks of each fraction at flow rate 0.5 ml/min, demonstrating that the column was well calibrated and that the purification condition were optimal. The high salt fraction CFII of the P-11 column showing bypass activity was loaded onto this column at the same flow rate to obtain 1 ml of each fraction. After incubation in XP-V (CTAG) alone,inCFII of P-11 or in Sephacryl fractions followed by digestion with MfeI/MboI/DpnI, replication of each fraction was verified by 0.8% agarose gel electrophoresis (figure 15). With nicked, linear and circular DNA bands appeared on the

gel, bypass replication was not seen in the negative control CTAG, but was seen in the positive control upon addition of the high salt fraction of P-11, the circular DNA band was seen on the gel. Morever, after addition of Sephacryl HR-200 fractions, bypass activity was observed in fractions No.37 to No.40 with fraction No.38 having the most bypass activity (48 mg of CFII application). Bypass activity was also seen in fractions No.57 to No. 66 (115.6 mg of CFII application). Cloudiness was observed during the purification procedure in fractions No.58 - No. 67.

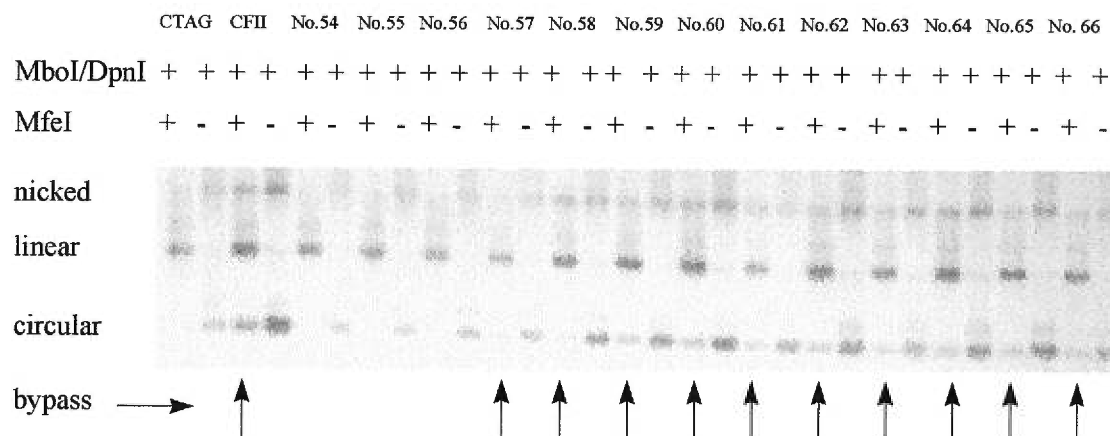

Figure 15. Sephacryl HR-200 column fraction from CFII of P-11 column complement XP-V defect on thymine dimer template.

The cloudy fraction had a higher concentration of protein (Bio-Rad assay) (table 4). After replication fraction No.60 showed a strong signal on the agarose gel, but did not separate well, as the protein peak corresponded only to that of thyroglobulin. The results of the replication bypass unit recovery of each fraction were the same as that observed on the agarose gel, where bypass replication activity started with the fraction contains

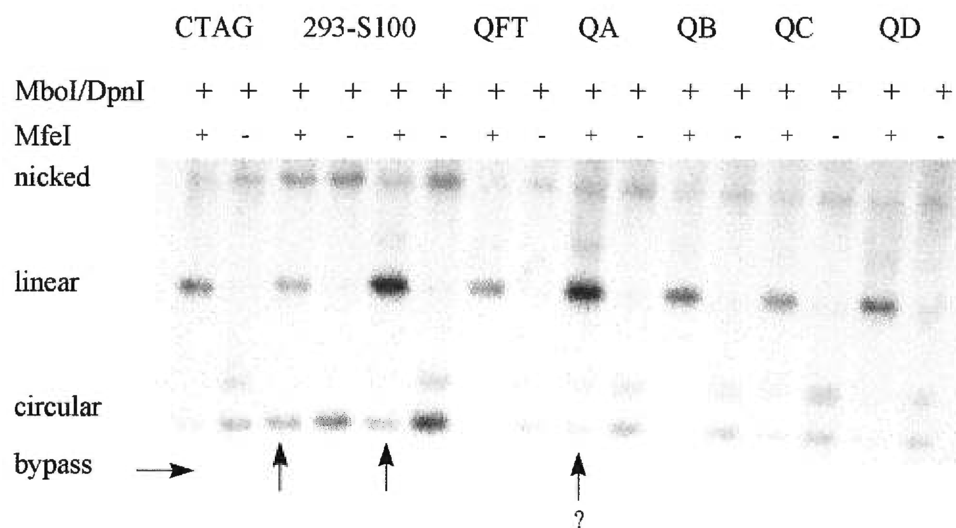

Figure 16. Q-Sepharose FF column fractions from CFII of P-11 appeared to have no bypass activity to complement XP-V defect on thymine dimer containing template.

DISCUSSION

IV.DISCUSSION

IV.1. DNA repair and XP

The study of DNA repair in human cells was limited for many years by the lack of available mutant cells defective in their response to DNA damage (Friedberg et al., 1995). In 1968 James Cleaver reported that cultured fibroblasts from the skin of patients with Xeroderma Pigmentosum (XP) were unable to carry out nucleotide excision repair following exposure to UV radiation (Cleaver, 1969, 1972). These observations were confirmed by Richard Setlow and his colleagues (Setlow et al., 1969). This was the first indication of a DNA repair defect associated with a hereditary human disease (Friedberg et al., 1995).

XP has been extensively reviewed in the literatures, and clinically is characterized chiefly by the early onset of severe photosensitivity of the exposed regions of the skin, eyes, and tongue. Among the ocular tissues, the eyelids, conjunctiva, and cornea are the most frequently affected, because they are subjected to substantial UV radiation exposure (Kraemer et al., 1987); Other features include a very high incidence of skin cancers and frequent neurological abnormalities (Friedberg et al.,1995). As a special group, XP-V was characterized by typical clinical features but with no apparent defect in nucleotide excision repair following exposure to UV radiation. XP-V cells have a clear defect in the replication of damaged DNA, and like classical XP cells, are hypermutable by a variety of DNA-damaging agents (Maher et al., 1976; Myhr et al., 1979).

Cleaver's observations on XP cells provided an impetus to examine the response to DNA-damaging agents of cells from a number of other hereditary human diseases, particularly those associated with chromosomal abnormalities or with an abnormally high incidence of neoplasia. In addition, the observation that human subjects suffering from XP are highly susceptible to malignant neoplasms caused by well-characterized mutagens and

carcino-genesis prompted further exploration of the relation between DNA damage, mutagenesis, and neoplastic transformation (Friedberg et al., 1995).

IV.2. *in vitro* SV40 DNA replication assay and 293 cell extracts

The small DNA tumor virus simian virus 40 (SV40) has served as an excellent model for many on the mechanism and control of gene expression in eukaryotic cells (Lebkowski et al., 1985). SV40 contains a covalently closed DNA genome of 5,243 base pairs that is maintained in a chromatin structure similar to the structure of cellular chromosomes (Prelich et al., 1987). Within the minichromosome lies a single origin of replication (ori) from which synthesis procedes bi-directionally and semi-conservatively around the molecule (Sundin and Varshavsky, 1981; DePamphilis and Wassarman, 1982; Weaver et al., 1985). The only virus-encoded protein required for replication is the SV40 large tumour antigen (large-T antigen) (Tegtmeyer, 1972). It is a multifunctional phosphoprotein of relative molecular mass of 90,000 (Mr 90 K) which is necessary for a number of processes, including transcriptional control (Tegtmeyer et al., 1975; Mayers et al., 1981; Hansen et al., 1981) and cellular transformation (Tooze, 1980) as well as DNA replication. The SV40 early region produces two protein products. One product (large-T antigen) is known to both repress early viral transcription and stimulate viral replication by binding to specific sites in the origin-promoter region (Lebkowski et al., 1985). SV40 does not replicate its DNA in most human cells very effectively (Stillman and Gluzman, 1985). Human cells are not natural hosts for SV40 virus, but they are permissive for replication of SV40 DNA (Ozer et al., 1981; Li and Kelly, 1984).

The main property of a cell- free system is to be able to replicate exogenous templates containing the SV40 origin of replication *in vitro* (Li and Kelly, 1985). As with replication of SV40 DNA in cell extracts prepared from monkey cells (Li and Kelly, 1984), cytoplasmic extracts prepared by hypotonic shock and Dounce homogenization from adenovirus-transformed human embryo kidney cells (human 293 cells) are capable of

supporting complete and efficient replication of SV40 DNA (Graham et al., 1977; Li and Kelly,1984; Lewis and Manley,1985). These cells express the adenovirus early-region E1A and E1B genes which are integrated in the chromosomal DNA and which could stimulate DNA synthesis directly or indirectly (Stillman and Gluzman,1985; Aiello et al., 1979). SV40 DNA replication was therefore carried out using soluble cell extracts from 293 cells.The 293 cells were adapted to growth in suspension culture rather than in monolayer culture, because of the large number of cells required for purification of replication factors. In this form, the cells can be maintained in suspension for at least 9 months and then frozen in liquid nitrogen to be reestablish in suspension culture when needed (Stillman and Gluzman, 1985).

Several lines of evidence indicate that the DNA synthesis observed in the cell-free system closely resemblesSV40 DNA replication in vivo. The system is completely dependent on the presence of the SV40 large T antigen. Extracts prepared from 293 cells support DNA replication in vitro (208). The results presented here have confirmed that 293 cells contain one or more factor(s) which stimulate DNA synthesis *in vitro,* especially in cytoplasmic extracts, since 293-S100 from different volume of 293 cells had bypass replication on T<>T template. Replication is dependent on the virus-encoded large T antigen, proceeds bidirectionally from the SV40 origin sequence, and requirs $MgCl_2$, ATP,deoxynucleoside triphosphates, and an energy-regenerating system (creatine phosphate and creatine phosphokinase). The product of the reaction mixture was migrated on an agarose gel as a full-length linear DNA after being digested with the approporiate restriction enzyme. The absence of substantial amounts of labeled species migrating more slowly than form III DNA (linear circular DNA) suggests that there were few partially replicated molecules containing a branch. In the absence of nuclear extract, the majority of reaction products migrate as a series of topoisomers that migrate from slightly slower than to slightly faster than the form II (nicked DNA) marker DNA for each plasmid, depending on the isomeric form (Stillman and Gluzman, 1985).

The development of cell extracts from suspension cells will expedite the identification of the cellular factor(s) required for initiation and elongation of DNA synthesis (Lewis and Manley, 1985). Exploitation of this system for replication of SV40 DNA should lead to the identification of cellular DNA replication factors, the elucidation of the mechanism of initiation,elongation,and termination of DNA synthesis, as well as the mechanism of resolution of DNA molecules. Ultimately, the complex regulation of DNA synthesis and the relationship between DNA replication and transcription from the SV40 origin- promoter region may be understood (Stillman and Gluzman, 1985). Since the replication of SV40 DNA only requires one virus encoded protein and is similar in mechanism to eukaryote chromosome replication, insight into the regulation of cellular DNA synthesis is an ultimate goal (Stillman and Gluzman, 1985). Also, 27-29 mers of the repair patches of excision of T<>T by an excision nuclease were not formed in XP cells or in extracts from XP cell lines. Therefore, it is reasonable to conclude that the enzyme which removes T<>T *in vitro* is the enzyme that is absent in XP cells (Svoboda et al., 1993). It is clear that the SV40 system is amenable to purification and will provide important insights into chromosomal replication (Murakami et al., 1986).

IV.3. High salt fraction of phosphate cellulose column had bypass activity.

Cellulose phosphate P-11 is a bifunctional cation exchanger containing both strong and weak acid groups based on an easter-linked orthophosphate functional group with normal pH range between 3 and 10. We treated the phosphocellulose column with NaOH and HCl to get the working pH range 3-11; as this is the basic requirement for protein complex purified from this type of column. The adsorbed components were eluted by increasing ionic concentration gradients (ie buffer or start buffer plus neutral salt). When possible, pH conditions should be selected so that the components of interest are not bound by ionic interactions (ie at a pH level above their isoelectric points). Subsequently the components of interest are eluted either by substrate elution (eg with ATP) or alternatively with increasing concentrations of phosphate buffer (manufacturer by Waltman).

Cell extract (S100) derived from repair-proficient human 293 cells were separated into two protein fractions (a flowthrough fraction CFI and a bound fraction CFII by phosphocellulose chromatography,and fractionated further to purify replication components, to monitor their ability to support simian virus 40 (SV40) DNA replication *in vitro* in the presence of SV40 large T antigen. Only high salt fraction CFII demonstrated bypass replication activity on a thymine dimer template in the SV40 DNA replication assay (Figure 13). This is in partial agreement with the work of others (Li et al., 1987; Prelich et al., 1987; Sugawara et al., 1996), where both cellular fraction CFI and CFII were required for the cell-free NER reactions to support SV40 DNA replication. The NER reactions with SV40 minichromosomes were completely dependent on CFI and CFII derived from 293-whole-cell extracts (Sugasawa et al., 1996). CFI can be replaced by two purified proteins, RPA and PCNA, since PCNA was recovered in CFI of P-11. CFI was shown to contain the cellular factors necessary for both the slow preinitiation step(s) of replication and for T antigen-dependent, origin-dependent unwinding (Li et al., 1987; Wold et al., 1987).

Since CFI did not demonstrate bypass activity in the *in vitro* DNA replication assay, it was not fractionated further. We continued however to fractionate CFII and to characterize the cellular proteins required to reconstitute SV40 DNA replication *in vitro*. After the initial fractionation, DNA polymerase activity along with many other proteins remained present in CFII. Evidence from both *in vivo* and *in vitro* experiments demonstrate that DNA polymerase α-primase is a major replicative polymerase in animal cells (Li and Kelly, 1984; Miller et al., 1985; Murakami et al., 1986; Wold et al., 1989). But the requirement of two polymerases (pol α and pol δ) in the minimal reconstituted DNA replication system leads to questions concerning the mechanism behind initiation and elongation. Since the primase associated with pol α is the only source of priming, it is evident that pol α/primase must be the first factor to initiate actual DNA synthesis. Reaction products generated by replication reactions without PCNA, the pol δ auxiliary factor, demonstrated that PCNA, and pol δ are required for leading strand synthesis (Prelich

and Stillman, 1988). Hence, pol δ must be primed by a DNA strand synthesized by pol α/primase (Melendy and Stillman, 1991).

Wold et al. (1989) purified DNA polymerase α-primase complex from CFII by chromatography on an anti-polymerase α immunoaffinity column and found that fraction CFIIA was absolutely required for SV40 DNA replication in reconstitution experiments. Very low levels of synthesis were observed in the absence of DNA poly-merase activity present in CFIIA (Wold et al., 1989). That suggests that fraction CFII was a protein complex, containing polymerase α-primase activity. It has been demonstrated previously that DNA chain elongation requires the presence of either topoisomerase I or topoisomerase II (Yang et al, 1987). The cytoplasmic extracts used to obtain CFI and CFII demonstrated only low levels of topoisomerase activity (Li et al., 1987). During the course of the fractionation procedure, topoisomerase I activity was removed from the active fraction. Thus, CFI contains no topoisomerase activity and CFII contains only small amounts of topoisomerase II activity (Wold et al., 1989).

Following the standard protein purification procedure, the high salt fraction CFII of P-11 contained polymerase α-primase, topoisomerase II, RFC, DNA ligase, and XPC-hHR23B, all of which are absolutely required for the SV40 DNA replication assay. These exciting results encourage us to further purify the components which complement the XP-V defect.

IV.4. Sephacryl HR-200 column fractions had bypass activity, but did not separate well.

Hiprep Sephacryl S-200 High Resolution gel filtration column provides a wide molecular range (from 5 to 1,500 kDa) for globular proteins. Sephacryl High Resolution is a cross-linked copolymer of allyl dextran and N,N-methylene-bisacrylamide. The narrow particle size distribution, together with steep selectivity curves, results in good preparative characteristics and maintains high resolution (manufacturer by Pharmacia).

Five peaks were obtained when the protein standard (Bio-Rad) was applied to the Sephacryl HR-200 column. The first and maximum peak was Thyroglobulin with a molecular weight of 670 KDa. This demonstrated that the column used, and the conditions under which it was used (flow rate, buffer, quantity of protein) were optimal for the protein purification. Loading of the high salt fraction CFII to the Sephacryl column under the same conditions resulted in only one peak fraction with bypass activity. This was because the high salt fraction CFII of the P-11 column which supports SV40 DNA replication and complements XP-V defect represented a large complex with a molecular weight of at least 670KDa. It was too early in the purification step for this sized chromatography to be used to purify the correcting factor(s) from normal human cell extracts. The adsorptive capacity of ion exchangers for proteins can be very high, but it depends on molecular size. Small proteins bind in amounts in excess of 100 $mgcm^{-3}$, but very large proteins ($>10^6$ MW) may not penetrate the exchanger and bind only to surfaces of the particles, resulting in very low capacities (Scopes, 1994). This problem can be circumvented by using another kind of column.

IV.5. Use of Phenyl sepharose column results in losss of bypass activity.

Phenyl Sepharose 6 Fast Flow (low sub) is a column with properties of hydrophobic interaction chromatography (HIC). The highly cross-linked beaded agarose matrices, which have excellent flow properties and high physical and chemical stabilities. Substances are separated based on the strength of the interactions of their hydrophobic groups with an uncharged matrix (manufacturer by Pharmacia). It is usually performed using moderately high concentration of salt in the adsorption buffer (salt promoted adsorption). Elution is achieved by a linear or stepwise decrease in concentration of the salt.

Bypass activity was no longer obtainable after application of the high bypass activity fraction of the Sephacryl HR-200 column to the Phenyl Sepharose low substance column. This was true for each peak fraction as well as for the mixture of each fraction (figure not shown). This suggests that the working conditions or Phenyl Sepharose

column itself were not suitable for the purification of replication components from the 293 cell extract. Factors such as type of ligand, the degree of substitution, the pH and the type and concentration of salt used during the adsorption stage have a profound effect on the overall performance (e.g. selectivity and capacity) of a HIC matrix. Other factors that affect HIC performance are temperature, detergents, polarity of solvents, type of matrix and ligand coupling chemistry (manufacturer by Pharmacia). Two buffers were attempted, buffer H with 1 M KCl and 1 M ammonium sulfate with 50 mM sodium phosphate. The pH of the buffer was also adjusted to enhance the hydrophobic interaction between the protein and the matrix. Different concentrations of salt were used to elute the protein. Despite these attempts, bypass activity was not observed. Clearly some of the proteins required for the SV40 DNA replication had been lost or degraded during the purification procedure. Since bypass activity was not obtained when the mixture of the different fractions was used in the replication assay. One possible reason is that DNA replication on T<>T template may have been inhibited by ammonium sulfate.

IV.6. Fractions of Q-Sepharose fast flow column appear to have bypass activity.

Hiload Q Sepharose Fast Flow is a strong anionic exchanger on which the groups are positively charged based on a rigid highly cross-linked beaded agarose. Sample molecules bind to the stationary charged groups, allow the others to pass through the column, and are eluted by competing charges in the enviroment. It is recommended to dilute viscous samples so that the maximum pressure (4.0 bar) is not exceeded under sample application and it is crucial to choose a buffer with the appropriate pH since the charge of a protein varies with pH (manufacturer by Pharmacia).

The high salt fraction CFII of the P-11 column was separated directly on a Q-Sepharose column into a 50 mM NaCl unbound fraction (QFT) and a number of bound fractions after step elutions with 150 mM (QA),250 mM (QB), 350 mM (QC), and 1M NaCl (QD). The DNA products of these reactions demonstrated that no fraction could replace CFII (figure 16), and even though fractions QFT or QA had bypass activity, this

activity was absent when the mixture of the five fractions was used (not shown). This suggests that the working conditions for this column was not optimal, since the optimal ion exchange separation conditions vary with each sample. Conditons such as pH and sample amount need to be adjusted.

Bypass replication of the T<>T lesion was impaired in XP-V extracts, while selective synthesis of Form I DNA from the undamaged strand by fork uncoupling was preserved in the XP-V cell extracts. Quantitation of the amount of fully replicated T<>T-containing daughter molecules relative to undamaged template in these extracts confirmed the unit recovery.

Following the standard protein purification procedure, we obtained a protein complex containing the replication components required for the SV40 DNA replication assay. Further experiments are needed to confirm and purify the components, but several of the known replication factors may be rationalized as candidates for XP-V correcting factor: 1) DNA polymerase delta catalyzes elongation of the primed template, and therefore interacts directly with T<>T during nucleotide insertion, 2) PCNA acts as a molecular clamp, reducing the rate at which polymerase delta dissociates from the primer end, increasing this processivity, 3) RF-C facilitates the loading of polymerase delta onto primer ends. These 3 candidates possess activities that influence the resultant rate of nucleotide insertion at the level of catalysis, polymerase dissociation, or polymerase binding. Another attractive possibility is the potential presence of an accessory factor which facilitates translesion synthesis analogous to the *E.coli* umuCD (Rajagopalan et al., 1992; Oda et al., 1996) and yeast REV systems (Nelson et al., 1996).

Isolation of the XP-V complementing factor should help to reveal the biochemical mechanisms of replication of UV-damaged DNA templates, and the mechanism of mutation induction by T<>T in human cells.

CONCLUSION

V. CONCLUSION

In conclusion, these studies demonstrate that normal human embryonic kidney 293 cells grow more preferably in suspension conditions than in monolayer culture. Successful growth of large quantities of human 293 cells in suspension conditions is necessary for preparation of human cell extracts to test for bypass activity. Fractionation of 293-S100 cells yielded partially purified complementing factor by a standard protein purification procedure. The effective an reproducible *in vitro* DNA replication assay system using a fraction purified by Phosphocellulose chromatography, demonstrated that 293 cells can complement XP-V defect. The Biochemical methods used confirm that it is feasible to purify the correcting factor(s) to complement the XP-V defect from human 293 cells. The studies on isolation of the XP-V correcting factor are important to isolate XP-V genes and to understand the roles played by these genes in the mechanisms of neoplastic transformation.

FUTURE WORKS

VI. FUTURE WORKS

It is acknowledged that there is still some experiments to do in the present studies to get better results. The following experiments must be helpful to purify correcting factor(s) from normal human cells:

1. Try more columns to complete the scheme of the XP-V correcting factor purification procedure from human 293 cell extracts and finally get purer complementing factor.

a. fraction CFII from cation exchanger chromatography (P-11) was a protein complex with bypass activity. We will further purify the protein complex by using anion exchanger chromatography (Q-sepharose or DEAE-Sephacel column), or by using Hydroxylapatite (hap) column, which can be equilibrated by buffer F + 100 mM KCl. And we will try to load CFII with step elution by continuing to mix 50 mM and 1M NaCl onto this column, but try different pH first, which definitely influence the purity of the sample.

b. Since Q-sepharose column is a good column to start with the fractionation of the samples. And we will try to load 293-S100 directly onto the Q-Sepharose column under the same conditons, after testing replication activity. We will further fractionated the protein complex on P-11 or DEAE -Sephacel column, which will be equalibrate d by buffer H + 15 mM KCl. It will get the successful results by changing the strategy.

c. Finally we will use the HR-200 Sepharose Chromatography in order to complete the scheme of protein purification.

3. Get the sequence from the purified protein to find out the mechanisms of XP-V defective in bypass replication. After purification through a few columns (above), we will check the purity of the proteins by running HPLC (high performance liquid chromatography) first, then by doing two dimentional gel to confirm the interest protein without interactions with others in order to obtain the sequence.

REFERENCES

VII. REFERENCES

Aboussekhra, A., Wood, R.D. (1994) Repair of UV-damaged DNA by mammalian cells and *Saccharomyces cerevisiae.* Curr. Opin. Genet. Dev. 4: 212-220

Aboussekhra A., Biggerstaff M., Shivji MK., Vilpo JA., Moncollin V., Podust VN., Protic M., Hubscher U., Egly JM., Wood RD.(1995) Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell 80(6):859-68

Aiello L., Guilfoyle R, Huebner K., Weinmann R.(1979) Adenovirus 5 DNA sequences present and RNA sequences transcribed in transformed human embryo kidney cells (HEK-AD-5 or 293). Virology 94:460-469.

Araki H., Ropp PA., Johnson AL., Johnston LH., Morrison A., Sugino A. (1992) DNA polymerase II, the probable homolog of mammalian DNA polymerase epsilon, replicates chromosomal DNA in the yeast Saccharomyces cerevisiae. EMBO J. 11(2):733-40.

Arase S., Kozuka T., Tanaka K., Ikenaga M., Takebe H. (1979) A sixth complementation group in xeroderma pigmentosum. Mutat.Res. 59(1):143-6.

Bhatia PK, Wang Z, Friedberg EC. (1996) DNA repair and transcription. Curr Opin Genet Dev 6(2):146-50.

Borowiec JA., Dean FB., Bullock PA., Hurwitz J.(1990) Binding and unwinding--how T antigen engages the SV40 origin of DNA replication. Cell 60(2):181-4.

Boyer J.C., Kaufmann W.K., Brylawski B.P., Cordeiro-Stone M.(1990) Defective post-replication repair in xeroderma pigmentosum variant fibroblasts. Cancer Res. 50(9):2593-8.

Bradford M.(1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding Anal. Biochem. 72:248-254.

Brash DE., Seetharam S., Kraemer KH., Seidman MM., Bredberg A. (1987) Photo-product frequency is not the major determinant of UV base substitution hot spots or cold spots in human cells. Proc.Natl.Acad.Sci.USA 84(11):3782-6.

Bravo R., Macdonald-Bravo M. (1985) Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. EMBO J. 4(3):655-661.

Bravo R., Frank R., Blundell P.A., MacDonald-Bravo H.(1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase- δ. Nature 326(2):515-517.

Bredberg, A., Kraemer, KH., Mseidman, MM.(1986) Restricted ultraviolet mutational spectrum in a shuttle vector propagated in xeroderma pigmentosum cells. Proc.Natl. Acad.Sci.USA 83(21):8273-8277.

Bredberg A., Kraemer K.H., Seidman M.M.(1986) Restricted ultraviolet mutational spectrum in a shuttle vector propagated in xeroderma pigmentosum cells. Proc.Natl.-Acad. Sci.USA 83(21):8273-7.

Bridges BA., Mottershead RP. (1976) Mutagenic DNA repair in *Escherichia coli*. III. Requirement for a function of DNA polymerase III in ultraviolet-light mutagenesis. Mol. Cen. Genet. 144(1):53-8.

Bridges B.A., Bates H.(1990) Mutagenic DNA repair in *Escherichia coli*. XVIII. Involvement of DNA polymerase III alpha-subunit (DnaE protein) in mutagenesis after exposure to UV light. Mutagenesis 5(1):35-8.

Brill S.J., Stillman B.(1989) Yeast replication factor-A functions in the unwinding of the S V40 origin of DNA replication. Nature 342(6245):92-95.

Broker T.R., Lehmann I.R.(1971) Branched DNA molecules: intermediates in T4 recombination. J.Mol.Biol 60(1):131-149.

Brourre F., A.Benoit, A.Sarasin(1989) Respective roles of pyrimidine dimer and pyrimidine (6-4) pyrimidone photoproducts in UV mutagenesis of simian virus 40 DNA in mammalian cells. J.Virol. 63(11):4520-4.

Buhl S.N., Stillman R.M., Setlow R.B., Regan J.D.(1972) DNA chain elongation and joining in normal human and xeroderma pigmentosum cells after ultraviolet irradiation. Biophys. J. 12(9):1183-1191.

Buhl S.N., Regan J.D.(1973) Repair endonuclease-sensitive sites in daughter DNA of ultraviolet-irradiated human cells. *Nature*(London) 246(5434):484.

Buhl SN., Regan JD.(1974) Effect of caffeine on postreplication repair in human cells. Biophys. J. 14:519-527.

Burgers P.M.(1991) Saccharomyces cerevisiae replication factor C. II. Formation and activity of complexes with the proliferating cell nuclear antigen and with DNA polymerases delta and epsilon. J.Biol.Chem. 266(33):22698-706.

Burk PG., Lutzner MA., Clarke DD., Robbins JH.(1971) Ultraviolet-stimulated thymidine incorporation in xeroderma pigmentosum lymphocytes. J.Lab.Clin.Med. 77(5):759-67.

Cadet J., Vigny P. (1990) in *Bioorganic photochemistry*, H.Morrison, Editor. 1990, John Wiley & Sons: New York.

Carty M.P., Hauser J., Levine A.S., Dixon K.(1993) Replication and mutagenesis of UV-damaged DNA templates in human and monkey cell extracts. Mol.Cell.Biol. 13(1):533-542.

Challberg M.D., Kelly T.J.(1982) Eukaryotic DNA replication: viral and plasmid model system. Annu.Rev.Biochem. 51:901-934.

Chou J.Y., Avila J., Martin R.G.(1974) Viral DNA synthesis in cells infected by temperature-sensitive mutants of simian virus 40. J.Virol. 14:116-124.

Cimino GD., Gamper HB., Isaacs ST., Hearst JE.(1985) Psoralens as photoactive probes of nucleic acid structure and function:organic chemistry, photochemistry, and Biochemistry. Annu.Rev.Biochem. 54:1151-93.

Cleaver J.E. (1968) Defective repair replication of DNA in xeroderma pigmentosum . Nature (Lond.) 218: 652-656

Cleaver J.E. (1969) Xeroderma pigmentosum: a human disease in which an initial stage of DNA repair is defective. Proc.Natl.Acad.Sci.USA 63:428-435.

Cleaver J.E., Thomas G.H.(1969) Single strand interruptions in DNA and the effects of caffeine in Chinese hamster cells irradiated with ultraviolet light. Biochem. Biophys.Res. Commun. 36(2):203-8.

Cleaver J.E. (1972) Xeroderma pigmentosum: variants with normal DNA repair and normal sensitivity to ultraviolet light . J. Invest. Dermatol. 58(3):124-8.

Cleaver J.E., Thomas G.H.,.Park S.D. (1979) Xeroderma pigmentosum variants have a slow recovery of DNA synthesis after irradiation with ultraviolet light. Biochim.Biophys.Acta 564(1):122-31.

Cleaver,J.E., Kraemer K.H. (1989) Xeroderma Pigmentosum in C.R.Scriver, A.L.Beaudet, W.S.Sly, and D.Valle(ed.), The metabolic basis of inherited disease, part II, p2949-2971, McGraw-Hill, New York.

Clingen, PH., Arlett CF., Roza, L., Mori, T., Nikaido, O., Green, MH-L. (1995) Induction of cyclobutane pyrimidine dimers, pyrimidine (6-4) pyrimidone photo-products,and Dewar Valence isomers by natural sunlight in normal human mononuclear cells. Cancer Res. 55:2245-2248.

Cordeiro-Stone M., Boyer JC., Smith BA., Kaufmann WK. (1986) Effect of benzo-[a]pyrene-diol-epoxide-I on growth of nascent DNA in synchronized human fibroblasts. Carcinogenesis 7(10):1775-81.

Cremisi C., Pignatti P.F., Croissant O., Yaniv M.(1976) Chromatin-like structures in polyma virus and simian virus 40 lytic cycle. J.Virol. 17:204-211.

Danna K.J., Nathans D.(1972) Bidirectional replication of simian virus 40 DNA. Proc.Natl. Acad.Sci.USA 69:3097-3100.

DePamphilis M.L., Wassarman P.M.(1980) Replication of eukaryotic chromosomes: a close-up of the replication fork. Annu.Rev.Biochem. 49:627-666.

DePamphilis M.L., Wassarman P.M. (1982) Qrganization and replication of papovavirus DNA 1982;37-114. In A.S.Kaplan(ed.), *Organization and replication of viral DNA*. CRC Press Inc., Boca Raton, Florida.

De Weerd-Kastelein E.A., Keijizer W., Bootsma D. (1972) Genetic heterogeneity of xeroderma pigmentosum demonstrated by somatic cell hybridization *Nature-New Biol.*, 238(81):80-83.

Drobetsky EA., Grosovsky AJ., Skandalis A., Glickman BW.(1989) Perspectives on UV light mutagenesis: investigation of the CHO *aprt* gene carried on a retroviral shuttle vector. Somat. Cell. Mol. Genet. 15(5):401-9.

Dumaz N., Drougard C., Sarasin A., Daya-Grosjean L.(1993) Specific UV-induced mutation spectrum in the p53 gene of skin tumors from DNA-repair-deficient xeroderma pigmentosum patients. Proc.Natl.Acad.Sci.USA. 90(22):10529-10533.

Eki T., Matsumoto T., Murakami Y., Hurwitz J.(1992) The replication of DNA containing the simian virus 40 origin by the monopolymerase and dipolymerase systems J.Biol.chem. 267(11):7284-94.

Fareed G.C., Garon C.F., Salzman N.P.(1972) Origin and direction of simian virus 40 deoxyribonucleic acid replication. J.Virol. 10:484-491.

Fiers W.,Contreras R,Haegeman, Rogiers R., van de Voorde A., van Heuversywn H., Van Herreweghe J., Volckaert G., Ysebaert M.(1978) The complete nucleotide sequence of SV40 DNA. *Nature*(London) 273:113-120.

Francis A.A., Regan J.D.(1986) Detection and repair of a UV-induced photosensitive lesion in the DNA of human cells. Mutat.Res. 165(3):151-157.

Friedberg E.C., et al.(1977) *Excision repair in mammalian cells and the current status of xeroderma pigmentosum pp.21-36.* In: Nichols WW, et al., ed. DNA repair processes. Miami, Symposium Specialists.

Friedberg, E.C. (1985), *DNA Repair*. 1985, New York: W.H. Freeman & Co.

Friedberg, E.C., Walker, G.C., Siede, W.(1994) *DNA Repair and Mutagenesis.* Chapter 1, pp.25-29.

Friedberg EC., Walker GC., Siede W. (1995) *DNA repair and mutagenesis* Chapter 14, pp.633.

Fornace A.J. Jr.(1983) Recombination of parent and daughter strand DNA after UV-irradiation in mammalian cells. *Nature*(London) 304(5926):552-554.

Fornace A.J.Jr., Kohn K.W., Kann Jr. H.E. (1976) DNA single-strand breaks during repair of UV damage in human fibroblasts and abnormalities of repair in xeroderma pigmentosum. Proc.Natl.Acad.Sci.USA 73(1):39-43.

Fujiwara, Y., Matsumoto, A., Ishihashi, M., Satoh, Y. (1987) Heritable disorders of DNA repair: Xeroderma pigmentosum and Fanconi's anemia. Curr.Probl.Dermatol 17:182-98.

Ganesan A.K.(1974) Persistence of pyrimidine dimers during post replication repair in ultraviolet light-irradiated Escherichia coli. J.Mol.Biol. 87:103-119.

Glazer P.M., S.N.Sarkar, W.C.Summers (1986) Detection and analysis of UV-induced mutations in mammalian cell DNA using a lambda phage shuttle vector. Proc.Natl. Acad.Sci.USA 83(4):1041-4.

Goulian M. Richards SH. Heard CJ. Bigsby BM.(1990) Discontinuous DNA synthesis by purified mammalian proteins [published erratum appears in J Biol Chem 1990 Dec 25; 265(36):22569]. J.Biol.Chem. 265(30):18461-71.

Graham f.J., Smiley J., Russell W.C., Nairu R.(1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J.Gen.Virol. 36:59-77.

Griffith J.D. (1975) Chromatin structure: deduced from a minichromosome. Science 187:1202-1203.

Griffiths TD, Ling SY (1991) Effect of UV light on DNA chain growth and replicon initiation in xeroderma pigmentosum variant cells. Mutagenesis 6(4):247- 51.

Grossman L., Thiagalingam S.(1993) Nucleotide excision repair, a tracking mechanism in search of damage. J. Biol. Chem. 268(23): 16871-16874.

Hagensee ME., Timme TL., Bryan SK., Moses RE.(1987) DNA polymerase III of *Escherichia coli* is required for UV and ethyl methanesulfonate mutagenesis. Proc.Natl.Acad.Sci.USA 84(12):4195-9.

Hall M., P.L.Grover(1990)*Polycyclic aromatic hydrocarbons: metabolism, activation and tumour initiation*,in Chemical Carcinogenesis and Mutagenesis I, C.S.Cooper, P.L.Grover, Editors. Springer-Verlag:Berlin. P.327-372.

Hansen U., Tenen D.G., Livingston D.M., Sharp P.A. (1981, 3 pt 2) T antigen repression of SV40 early transcription from two promoters. Cell 27:603-613.

Hauser J., Seidman MM., Sidur K., Dixon K. (1986) Sequence specificity of point mutations induced during passage of a UV-irradiated shuttle vector plasmid in monkey cells. Mol.Cell.Biol. 6(1):277-85.

Hay R.T., DePamphilis M.L.(1982) Initiation of SV40 DNA replication *in vivo*: location and structure of 5'end of DNA synthesized in the *ori* region. Cell 28:767-779.

Hessel A, Siegle RJ, Mitchell DL, Cleaver JE.(1992) Xeroderma pigmentosum variant with multisystem involvement. Arch. Dermatol. 128(9):1233-7.

Higgins N.P., Kato K., Strauss B. (1976) A model for replication repair in mammalian cells. J.Mol.Biol 101(3):417-425.

Hoeijmakers J.H.J. (1993) Nucleotide excision repair I: from *E.coli* to yeast. Trends Genet. 9(5):173-177.

Hoeijmarkers J.H., Bootsma D.(1994) DNA repair. Incisions for excision [news; comment][published erratum appears in Nature 1994 Nov 17;372(6503):226]. Nature 371(6499):654-5.

Hoffmann JS, Pillaire MJ, Maga G, Podust V, Hubscher U, Villani G.(1995) DNA polymerase beta bypasses in vitro a single d(GpG)-cisplatin adduct placed on codon 13 of the HRAS gene. Proc.Natl.Acad.Sci.USA 92(12):5366-60.

Holliday R.(1974) Molecular aspects of genetic exchange and gene conversion. Genetics 78:273-287.

Howard-Flanders P., Rupp W.D., Wilkins B.M.(1968) DNA replication and recombination after UV irradiation. Cold Spring Harb. Symp.Quant. Biol. 33: 195-207.

Hsia HC., Lebkowski JS., Leong PM., Calos MP., Miller JH. (1989) Comparison of ultra-violet irradiation-induced mutagenesis of the *lacI* gene in *Escherichia coli* and in human 293 cells. J.Mol.Biol. 205(1):103-13.

Hurwitz J., Dean FB., Kwong AD., Lee SH.(1990) The *in vitro* replication of DNA containing the SV40 origin. J. Biol. Chem. 265(30):18043-046.

Ishimi Y. Claude A. Bullock P. Hurwitz J.(1988) Complete enzymatic synthesis of DNA containing the SV40 origin of replication. J.Biol.Chem. 263(36):19723-33.

Iyer V.N., Rupp W.D.(1971) Usefulness of benzoylated naphthoylated DEAE-cellulose to distinguish and fractionate double-stranded DNA bearing different extents of single-stranded regions. Biochim.Biophys.Acta 228(1):117-26.

Jimbo T, Ichihashi M, Mishima Y, Fujiwara Y.(1992) Role of excision repair in UVB-induced depletion and recovery of human epidermal Langerhans cells. Arch.Dermatol. 128(1):61-7.

Kaufmann W.K., Cleaver J.E., Painter R.B.(1980) Ultraviolet radiation inhibits replicon initiation in S phase human cells. Biochim. Biophys. Acta 608(1):191-5.

Kaufmann W.K., Cleaver J.E.(1981) Mechanisms of inhibition of DNA replication by ultraviolet light in normal human and xeroderma pigmentosum fibroblasts. J.Mol.Biol. 149(2):171-87.

Keijzer W., Jaspers NG., Abrahams PJ., Taylor AM., Arlett CF., Zelle B., Takebe H., Kinmont PD., Bootsma D.(1979) A seventh complementation group in excision-deficient xeroderma pigmentosum. Mutat.Res. 62(1):183-90.

Kelly T.J.(1984) in *The adenoviruses*, ed.Gimsberg, H.(Plenum, New York), 271-307.

Kelly T.J., Wold M.S., Li J.J. (1988) Initiation of viral DNA replication. Adv.Virus Res. 34:1-42.

King S.A.., Wilson S.J., Farber R.A., Kaufmann W.K., Cordeiro-Stone M. (1995) Xeroderma pigmentosum variant: generation and characterization of fibroblastic cell lines transformed with SV40 large T antigen. Exp. Cell Res. 27(1):100-108

Kraemer KH., De Weerd-Kastelein EA., Robbins JH., Keijzer W., Barrett SF., Petinga RA., Bootsma D. (1975) Five complementation groups in xeroderma pigmentosum. Mutat.Res. 33(2-3):327-40.

Kraemer K.H., Lee M.M., Scotto J. (1984) DNA repair protects against cutaneous and internal neoplasia: evidence from xeroderma pigmentosum. Carcinogenesis 5(4):511-4.

Kraemer K.H., Lee M.M., Scotto J. (1987) Xeroderma pigmentosum. Cutaneous, ocular, and neurologic abnormalities in 830 published cases. Arch. Dermatol. 123(2):241-50.

Kunieda, T., Witkop, B. (1971) Hydrogenolysis and stereochemistry of photodimers of thymine and thymidine. J. Am.Chem. Soc. 93(14):3493-9.

Lavin MF. (1978) Postreplication repair in mammalian cells after ultraviolet irradiation—A model. Biophys. J 23:247-256.

Leaver J.E., Thomas G.H., Park S.D.(1979) Xeroderma pigmentosum variants have a slow recovery of DNA synthesis after irradiation with ultraviolet light. Biochim.Biophys. Acta (1);564:122-131.

Lebkowski JS., Clancy S., Calos MP.(1985) Simian virus 40 replication in adenovirus-transformed human cells antagonizes gene expression. Nature. 317 (6033):169-71.

Lee SH., Kwong AD., Pan ZQ., Hurwitz J.(1991) Studies on the activator 1 protein complex, an accessory factor for proliferating cell nuclear antigen-dependent DNA polymerase delta. J.Biol.Chem. 266(1):594-602.

Lee C.S. , Davis R.W., Davidson N.(1970) A physical study by electron microscopy of the terminally reptitious, circularly permuted DNA from the coliphage particles of *Escherichia coli* 15. J.Mol.Biol 1970;48(1):1-22.

Lehmann A.R.(1972) Postreplication repair of DNA in ultraviolet-irradiated mammalian cells. J. Mol. Biol 66:319-37.

Lehmann A.R.(1972) Post-replication repair of DNA in ultraviolet-irradiated mammalian cells. No gaps in DNA synthesized late after ultraviolet irradiation. Eur. J. Biochem. 31(3):438-45.

Lehmann A.R.(1974) Postreplication repair of DNA in mammalian cells. Life Sci. 15(12):2005-16.

Lehman A.R., Kirk-Bell S., Arlett C.F., Paterson M.C., Lohman P.H.M., de Weerd-Kastelein E.A., Bootsma D.(1975) Xeroderma pigmentosum cells with normal levels of excision repair have a defect in DNA synthesis after UV-irradiation. Proc.Natl. Acad. Sci.USA 72(1):219-223.

Lehamann A.R., Kirk-Bell S., Jaspers N.G.(1977) Postreplication repair in normal and abnormal human fibroblasts pp.203-15 In: Nichols WW, et al., ed. DNA repair processes. Miami, Symposium Specialists.W1 CE1295 v.4 1977.

Lehmann A.R., Kirk-Bell S. (1978) Pyrimidine dimer sites associated with the daughter DNA strands in UV-irradiated human fibroblasts. Photochem. Photobiol. 27(3):297-307.

Léveillard T., Andera L., Bissonnette N., Schaeffer L., Bracco L., Egly JM.,Wasylyk B. (1996) Functional interactions between p53 and the TFIIH complex are affected by tumor-associated mutations. EMBO J. 15(7):1615-1624.

Ley R.D.(1973) Postreplication repair in an excision-defective mutant of Escherichia coli: ultraviolet light-induced incorporation of bromodeoxyuridine into parental DNA. Photochem.Photobiol. 18(2):87-95.

Lewis E.D., Manley J.L.(1985) Repression of simian virus 40 early transcription by viral DNA replication in human 293 cells. Nature 317(6033):172-5.

Li C., Cao LG., Wang YL., Baril EF.(1993) Further purification and characterization of a multienzyme complex for DNA synthesis in human cells. J.Cell.Biochem. 53(4):405-19.

Li J.J., Kelly T.J.(1984) Simian virus 40 DNA replication *in vitro*. Proc.Natl. Acad.Sci.U.S.A 81(22):6973-6977.

Li, JJ., Kelly, TJ. (1985) Simian virus 40 DNA replication *in vitro*: specificity of initiation and evidence for bidirectional replication. Mol. Cell. Biol. 5(6):1238-1246.

Li, JJ., Peden KW.-C., Dixon RA.-F., Kelly T.(1986) Functional Organization of the simian virus 40 origin of DNA replication. Mol. Cell. Biol. 6(4):1117-1128.

Li,J.J., Wold M.S., Kelly T.J.(1987) in *Mechanisms of DNA Replication and Recombination* (Kelly T.J. and McMakerg R., eds) pp.289-301, Academic Press, Orlando, FL.

Lin YL, Shivji MK, Chen C, Kolodner R, Wood RD, Dutta A (1998) The evolutionarily conserved zinc finger motif in the largest subunit of human replication

protein A is required for DNA replication and mismatch repair but not for nucleotide excision repair. J. Biol. Chem. 273(3):1453-61.

Livneh Z., Cohen-Fix O., Skaliter R., Elizur T. (1993) Replication of damaged DNA and the molecular mechanism of ultraviolet light mutagenesis. Crit.Rev.Biochem. Mol. Biol. 28(6):465-513.

Loor G, Zhang SJ, Zhang P, Toomey NL, Lee MY. (1997) Identification of DNA replication and cell cycle proteins that interact with PCNA. Nucleic Acids Res. 25(24): 5041-6.

Madsen P., Celis J.E.(1985) S-phase patterns of cyclin (PCNA) antigen staining resemble topographical patterns of DNA synthesis. A role for cyclin in DNA replication? FEBS Lett. 193(1):5-11.

Maher VM., Ouellette LM., Curren RD., McCormick JJ.(1976) Frequency of ultra-violet light-induced mutations is higher in xeroderma pigmentosum variant cells than in normal human cells. *Nature* (London) 261(5561):593-5.

Maniatis, Sambrook, Fritsch(1985) *Molecular Cloning* 2nd edition, Chapter 4 - 4.38.

Masamune Y., Richardson CC.(1971) Strand displacement during deoxyribonucleic acid synthesis at single strand breaks. J.Biol.Chem 246(8):2692-2701.

Masutani C., Sugasawa K., Yanagisawa J., Sonoyama T., Ui M., Enomoto T., Taikio K., Tanaka K., van der Spek P.J., Bootsma D., Hoeijmakers J.H.J., Hanaoka F. (1994) Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homolog of yeast RAD23. EMBO J. 8:1831-1843.

Matsuda T, Saijo M, Kuraoka I, Kobayashi T, Nakatsu Y, Nagai A, Enjoji T, Masutani C, Sugasawa K, Hanaoka F, et al (1995) DNA repair protein XPA binds replication protein A (RPA). J. Biol. Chem. 270(8):4152-7.

Mayers R.M., Rio D.C., Robbins A.K., Tjian R.(1981) SV40 gene expression is modulated by the cooperative binding of T antigen to DNA. Cell 25:373-384.

Meneghini R., Hanawalt P.C.(1975) Postreplication repair in human cells: on the presence of gaps opposite dimers and recombination. Basic Life Sci. 5B:639-642.

Meneghini R. (1976) Gaps in DNA synthesized by ultraviolet light-irradiated WI38 human cells. Biochim. Biophys.Acta 425:419-427.

Meselson M.S., Radding C.M.(1975) A general model for genetic recombination. Proc.Natl.Acad.Sci.USA 72:358-361.

Melendy T., Stillman B. (1991) Purification of DNA polymerase δ as an essential simian virus 40 DNA replication factor. J. Biol. Chem. 266 (3): 1942-1949.

Miller M.R., Ulrich R.G., Wang T.S-F., Korn D.(1985) Monoclonal antibodies against human DNA polymerase-alpha inhibit DNA replication in permeabilized human cells. J.Biol.Chem. 260(1):134-138.

Misra, RR., Vos, JM-H.(1993) Defective replication of psoralen adducts detected at the gene-specific level in xeroderma pigmentosum variant cells. Mol. Cell. Biol. 3(2):1002-1012.

Mu D., Park CH., Matsunaga T., Hsu DS., Reardon JT., Sancar A. (1995) Reconstitution of human DNA repair excision nuclease in a highly defined system. J.Biol.Chem. 1995; 270(6):2415-8.

Murakami Y., Wobbe C.R., Weissbach L., Dean F.B., Hurwitz J. (1986) Role of DNA polymerase alpha and DNA primase in simian virus 40 DNA replication *in vitro*. Proc.Natl.Acad.Sci.U.S.A 83(9):2869-2873.

Myhr B.C., Turnbull D., DiPaolo J.A.(1979) Ultraviolet mutagenesis of normal and xeroderma pigmentosum variant human fibroblasts. Mutat. Res. 63:341-353.

Napolitano RL, Lambert IB, Fuchs RP.-P (1997) SOS actors involved in translesion synthesis. Proc.Natl.Acad.Sci.USA 94:5733-5738.

Nelson J.R., Lawrence C.W., Hinkle D.C. (1996) Thymine-thymine dimer bypass by yeast DNA polymerase zeta. Science 272(5268):1646-1649.

Oda N., Levin J.D., Spoonde A.Y., Frank E.G., Levine A.S., Woodgate R., Ackerman E.J.(1996) Arrested DNA replication in Xenopus and release by *Escherichia coli* mutagenesis proteins. Science 272(5268):1644-1646.

Ozer H.L., Slator M.L., Dermody J.J., Mandel M.(1981) Replication of simian virus 40 DNA in normal human fibroblasts and in fibroblasts from xeroderma pigmentosum. J.Viol. 39(2):481-489.

Pan ZQ, Park CH, Amin AA, Hurwitz J, Sancar A (1995) Phosphorylated and unphos- phorylated forms of human single-stranded DNA-binding protein are equally active in simian virus 40 DNA replication and in nucleotide excision repair. Proc.Natl.Acad.Sci.U.S.A 92(10):4636-40

Park S.D., Cleaver J.E. (1979) Recovery of DNA synthesis after ultraviolet irradiation of xeroderma pigmentosum cells depends on excision repair and is blocked by caffeine. Nucleic Acids Res. 6(3):1151-9.

Podust V.N., Hubscher U.(1993) Lagging strand DNA synthesis by calf thymus DNA polymerases alpha, beta, delta and epsilon in the presence of auxiliary proteins. Nucleic Acids Res. 21(4):841-6.

Prelich G., Kostura M., Marshak D.R., Mathews M.B., Stillman B.(1987a) The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication *in vitro*. *Nature* 326(6112):471-475.

Prelich G., Tan C-K., Kostura M., Marshak D.R., Mathews M.B., So A.G., Downey K.M., Stillman B.(1987b) Functional identity of proliferating cell nuclear antigen and a DNA polymerase-delta auxiliary protein. *Nature* 326(6112):517-520.

Prelich G., Stillman B.(1988) Coordinated leading and lagging strand synthesis during SV40 DNA replication *in vitro* requires PCNA. Cell 53(1):117-126.

Protic-Sabljic M., Tuteja N., Munson PJ., Hauser J., Kraemer KH., Dixon K. (1986) UV light-induced cyclobutane pyrimidine dimers are mutagenic in mammalian cells. Mol.Cell.Biol. 6(10):3349-56.

Radding C.(1973) Molecular mechanism in genetic recombination. Annu.Rev.Genet. 7:87-111.

Rajagopalan M., Lu C., Woodgate R., O'Donnel M., Goodman M.F., Echols H. (1992) Activity of the purified mutagenesis proteins UmuC, UmuD', and RecA in replicative bypass of an abasic DNA lesion by DNA polymerase III Proc.Natl.Acad. Sci.USA 89(22):10777-10781.

Reardon JT., Spielmann P., Huang JC., Sastry S., Sancar A., Hearst JE. (1991) Removal of psoralen monoadducts and crosslinks by human cell free extracts. Nucleic Acids Res. 19(17):4623-9.

Reddy V.B., Thimmappaya B., Dhar R., Subramanian K.N., Zain B.S., Pan J., Ghosh P.K., Celma M.L., Weissman S.M.(1979) The genome of SV40. Science 200:494-502.

Robbins J.H.(1988) Xeroderma pigmentosum. Defective DNA repair causes skin cancer and neurodegeneration [clinical conference]. I.Am.Med.Assoc. 260(3):384-388.

Rossignol M, Kolb-Cheynel I, Egly JM.(1997) Substrate specificity of the cdk-activating kinase (CAK) is altered upon association with TFIIH. EMBO J. 16 (7):1628-37.

Rude J.M, Friedberg E.C.(1975) Semi-conservative deoxyribonucleic acid synthesis in unirradiated and ultraviolet-irradiated xeroderma pigmentosum and normal human skin fibroblasts. Mutat.Res 33:327-340.

Rupp W.D., Howard-Flanders P.(1968) Discontinuities in the DNA synthesized in an excision- defective strain of *Escherichia coli* following ultraviolet irradiation. J. Mol. Biol. 31(2):291-304.

Rupp W.D., Wilde C.E., III, Reno, D.L., Howard-Flanders P.(1971) Exchanges between DNA strands in ultraviolet-irradiated *Escherichia coli*. J.Mol.Biol 61:25-44.

Sambrook, J. et al., eds. (1989) *Molecular cloning: a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, p. 1.32.

Sancar A.(1995) Excision repair in mammalian cells. J.Biol. Chem. 270(27):15915-18.

Sancar A. (1996) DNA excision repair. Annu.Rev. Biochem. 65:43-81

Sarasin A., Stary A.(1997) Human cancer and DNA repair-deficient diseases. Cancer Detection & Prevention 21(5):406-11.

Scopes RK.(1994) *Protein purification-Principles and practice*, Third edition, pp166.

Sedgwick S.G.(1975) Genetic and kinetic evidence for different types of postreplication repair in *Escherichia coli* B. J.Bacteriol. 123(1):154-161.

Seguin LR,Ganges MB,Tarone RE, Robbins JE.(1992) Skin cancer and chromosomal aberrations induced by ultraviolet radiation. Evidence for lack of correlation in xeroderma pigmentosum variant and group E patients. Cancer Genet.Cytogenet. 60(2):111-6.

Selby, CP., Drapkin, R., Reinberg, D., Sancar A. (1997) RNA polymerase II stalled at a thymine dimer: footprint and effect on excision repair. Nucleic Acids Res. 25(4):787-793.

Selkirk, J.K., et al.(1982) *Species variance in the metabolic activation of polycyclic hydrocarbons*, in Mechanisms of Chemical Carcinogenesis, C.C.Harris, P.A.Cerutti, Editors. Alan R.Liss, Inc.: New York.

Seroz T, Hwang JR, Moncollin V, Egly JM.(1995) TFIIH: a link between transcription, DNA repair and cell cycle regulation. Curr. Opin.Gen.Dev. 5(2):217-21.

Setlow R.B.(1966) Cyclobutane-type pyrimidine dimers in polynucleotides. Science 153(734):379-386.

Setlow R.B., Regan J.D., German J., Carrier W.L.(1969) Evidence that xeroderma pigmentosum cells do not perform the first step in the repair of ultraviolet damage to their DNA. Proc.Natl.Acad.Sci.USA 64(3):1035-1041

Shivji Mk.K., Eker AP.-M., Wood RD.(1994) DNA repair defect in Xeroderma Pigmentosum group C and complementing factor from Hela cells. J.Biol.Chem. 269(36):22749-22757.

Sibghatullah., Husain I., Carlton W., Sancar A. (1989) Human nucleotide excision repair in vitro: repair of pyrimidine dimers, psoralen and cisplatin adducts by HeLa cell-free extract. Nucleic Acids Res. 17(12):4471-84.

Smith K.C., Meun D.H.C. (1970) Repair of radiation-induced damage in *Escherichia coli*. I. Effect of rec mutations on post-replication repair of damage due to ultraviolet radiation. J.Mol.Biol. 51(3):459-472.

Stillman BW. (1983) The replication of adenovirus DNA with purified proteins. Cell 35(1):7-9.

Stillman BW., Gluzman Y.(1985) Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. Mol. Cell. Biol. 5(8):2051-2060.

Sugasawa K., Masutani C., Uchida A., Maekawa T., VAN DER Spek PJ., Bootsma D., Hoeijmakers JH.-J., Hanaoka F.(1996) HHR23B, a human Rad23 homolog, stimulates XPC protein in nucleotide excision repair *in vitro*. Mol.Cell. Biol. 16(9):4852-4861.

Sugasawa K., Ng JM.-Y., Masutani C., Maekawa T., Uchida A., vander Spek PJ., AP.-M.Eker, Rademakers S., Visser C., Aboussekhra A., Wood RD., Hanaoka F., Bootsma D., Hoeijmakers JH.-J.(1997) Two human homologs of Rad23 are functionally

interchangeable in complex formation and stimulation of XPC repair activity. Mol.Cell. Biol. 17(12):6924-6931.

Sundin O., Varshasky A.(1981) Arrest of segregation leads to accumulation of highly intertwined catenated dimers: dissection of the final stages of SV40 DNA replication. Cell 25(3):659-669.

Svobda DL., Taylor JS., Hearst JE., Sancar A.(1993) DNA repair by eukaryotic nucleotide excision nuclease- Removal of thymine dimer and psoralen monoadduct by Hela cell-free extract and of thymine dimer by Xenopus Laevis oocytes . J.Biol. Chem. 268(3):1931-1936.

Svoboda D.L., Vos J.M.(1995) Differential replication of a single, UV-induced lesion in the leading or lagging strand by a human cell extract: fork uncoupling or gap formation. Proc.Natl.Acad.Sci.USA 92(26):11975-9.

Svoboda DL., Briley LP., Vos JM.-H.(1998) Defective bypass replication of a site-specific UV-induced leading strand thymine dimer in Xeroderma pigmentosum variant cell extract. Cancer Res. 58(11):2445-8.

Szymkowski D.E., Lawrence C.W.,. Wood R.D(1993) Repair by human cell extracts of single (6-4) and cyclobutane thymine-thymine photoproducts in DNA. Proc.Natl. Acad.Sci.USA 90(21):9823-7.

Taylor J.S., Brockie I.R.(1988) Synthesis of a trans-syn thymine dimer building block. Solid phase synthesis of CGTAT[t,s]TATGC. Nucleic Acids Res. 16(11):5123-36.

Tegtmeyer P.(1972) Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. 10(4):591-598.

Tegtmeyer P., Schwartz M., Collins J.K., Rundell K.(1975) Regulation of tumor antigen synthesis by simain virus 40 gene A. J.Virol. 16(1):168-178.

Tjian R.(1981) T antigen binding and the control of SV40 gene expression. Cell 26(1 pt 1):1-2.

Tooze J. (1980) *Molecular Biology of Tumour Viruses*, 2nd edn. Part 2 (Cold Spring Harbor Laboratory, New York)

Tsurimoto T., Melendy T., Stillman B.(1990) Sequential initiation of lagging and leading strand synthesis by two different polymerase complexes at the SV40 DNA replication origin. *Nature* (London) 346(6284):534-9.

Tsurimoto T., Stillman B. (1990) Functions of replication factor C and proliferating-cell nuclear antigen: functional similarity of DNA polymerase accessory proteins from human cells and bacteriophage T4. Proc.Natl.Acad.Sci.USA 87:1023-27.

Tsurimoto T., Stillman B. (1991a) Replication factors required for SV40 DNA replication *in vitro*. I. DNA structure-specific recognition of a primer-template junction by eukaryotic DNA polymerases and their accessory proteins. J.Biol.Chem. 266: 1950-1960.

Tsurimoto T., Stillman B.(1991b) Replication factors required for SV40 DNA replication *in vitro*. II. Switching of DNA polymerase alpha and delta during initiation of leading and lagging strand synthesis. J.Biol.Chem. 266:1961-1968.

Turchi J.J., Bambara R.A.(1993) Completion of mammalian lagging strand DNA replication using purified proteins. J.Biol.Chem. 268(20):15136-41.

Uhlmann F, Gibbs E, Cai J, O'Donnell M, Hurwitz J. (1997) Identification of regions within the four small subunits of human replication factor C required for complex formation and DNA replication. J.Biol.Chem. 272(15):10065-71.

Vos J.-M.H.(1995) in *DNA Repair Mechanisms: Implications for Human Disease and Cancer*, ed.Vos J.-M.H.(Landes, Austin, TX), Chap.9, pp 187-218.

Waga S., Bauer G., Stillman B.(1994) Reconstitution of complete SV40 DNA replication with purified replication factors. J.Biol.Chem. 269(14):10923-34.

Wang S.Y., ed.(1976) *Photochemistry and photobiology of nucleic acids*, Academic Press, Inc.: New York

Wang YC, Maher VM, McCormick JJ (1991) Xeroderma pigmentosum variant cells are less likely than normal cells to incorporate dAMP opposite photoproducts during replication of UV-irradiated plasmids. Proc.Natl.Acad.Sci.USA 88(17):7810-4.

Wang YC, Maher VM, Mitchell DL, McCormick JJ.(1993) Evidence from mutation spectra that the UV hypermutability of xeroderma pigmentosum variant cells reflects abnormal, error-prone replication on a template containing photoproducts. Mol.Cell.Biol. 13(7):4276-83.

Watanabe M, Maher VM, McCormick JJ.(1985) Excision repair of UV- or benzo-[a]pyrene diol epoxide-induced lesions in xeroderma pigmentosum variant cells is 'error free'. Mutation Res. 146(3):285-94.

Waters HL, Seetharam S, Seidman MM, Kraemer KH. (1993) Ultraviolet hyper-mutability of a shuttle vector propagated in xeroderma pigmentosum variant cells. J. Invest. Dermatol. 101(5):744-8.

Weaver D.T., Fields-Berry S.C., DePamphilis M.L. (1985) The termination region for SV40 DNA replication directs the mode of separation for the two sibling molecules. Cell 41(2):565-575.

Weinberg DH., Collins KL., Simancek P., Russo A., Wold MS., Virshup DM., Kelly TJ.(1990) Reconstitution of simian virus 40 DNA replication with purified proteins. Pro.Natl.Acad.Sci.USA 87(22):8692-6.

Wold M.S., Li J.J., Kelly T.J.(1987) Initiation of simian virus 40 DNA replication *in vitro*: large-tumor-antigen- and origin-dependent unwinding of the template. Proc.Natl.Acad.Sci.U.S.A. 84(11):3643-3647.

Wold M.S., Kelly T.J.(1988) Purification and characterization of replication protein A, a cellular protein required for *in vitro* replication of simian virus 40 DNA. Proc.Natl. Acad.Sci.USA 85(8):2523-2527.

Wold MS., Weinberg DH., Virshup DM., Li JJ., Kelly TJ.(1989) Identification of cellular proteins required for simian virus 40 DNA replication. J.Biol.Chem. 264(5):2801-2809.

Wold MS.(1997) Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu.Rev. Biochem. 66:61-92.

Wood R.D., Robins P., Lindahl T.(1988) Complementation of the xeroderma pigmentosum DNA repair defect in cell-free extracts. Cell 53(1):97-106.

Wood R.D. (1995) Proteins that participate in nucleotide excision repair of DNA in mammalian cells.Philosophical Transactions of the Royal Society of London - Series B: Biol. Sci. 347(1319):69-74.

Wood RD.(1997) Nucleotide excision repair in mammalian cells. J. Biol. Chem. 272(38):23465-68.

Yang L., Woold M.S., Li, J.J., Kelly T.J., Liu L.F. (1987) Roles of DNA topoisomerases in simian virus 40 DNA replication *in vitro*. Proc.Natl.Acad.Sci.U.S.A. 84(4):950-954.

Youngs D.A., Smith K.C.(1976) Genetic control of multiple pathways of post-replicational repair in uvrB strains of *Escherichia coli* K-12. J.Bacteriol. 125(1):102-110.